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Risk assessment for creatine monohydrate ☆

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Abstract

Creatine monohydrate (creatine) has become an increasingly popular ingredient in dietary supplements, especially sports nutrition products. A large body of human and animal research suggests that creatine does have a consistent ergogenic effect, particularly with exercises or activities requiring high intensity short bursts of energy. Human data are primarily derived from three types of studies: acute studies, involving high doses (20 g/d) with short duration ($\leqslant$1 week), chronic studies involving lower doses (3–5 g/d) and longer duration (1 year), or a combination of both. Systematic evaluation of the research designs and data do not provide a basis for risk assessment and the usual safe Upper Level of Intake (UL) derived from it unless the newer methods described as the Observed Safe Level (OSL) or Highest Observed Intake (HOI) are utilized. The OSL risk assessment method indicates that the evidence of safety is strong at intakes up to 5 g/d for chronic supplementation, and this level is identified as the OSL. Although much higher levels have been tested under acute conditions without adverse effects and may be safe, the data for intakes above 5 g/d are not sufficient for a confident conclusion of long-term safety.

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Keywords: Creatine; Upper Level of Intake (UL); Observed Safe Level (OSL)

1. Introduction

First discovered in 1832, creatine is a naturally occurring amino acid-like compound made in the liver, kidneys and pancreas from the essential amino acids arginine, glycine and methionine (Balsom et al., 1994). In humans, over 95% of the total creatine content is located in skeletal muscle. A 70 kg male possess approximately 120 g of total creatine with a daily turnover estimated to be around 2 g. Part of this turnover can be replaced through exogenous sources of creatine in foods, including meat, fish and poultry (Balsom et al., 1994). In its phosphorylated intracellular form as creatine phosphate, it provides the high energy phosphate for adenosine triphosphate.

As a dietary supplement, creatine is available in powdered, tablet and liquid forms as primarily creatine monohydrate, the only form having been included in published, randomized, controlled trials. In the last 10 years, nearly 70 randomized, controlled trials have been conducted on or with creatine, with the majority examining creatine's performance-enhancing benefits. The majority of these clinical trials have found beneficial effects from creatine supplementation, particularly during short, repeated bursts of high-intensity activity (Bemben and Lamont, 2005). Recent research efforts have also focused on the potential benefits of creatine use in patients coping with certain neuromuscular disorders (Persky and Brazeau, 2001).

Initial recommendations for creatine use stemmed from early research using 5–7 days of "loading" with 20–30 g per day (divided into 4–6 equal, 5 g doses), resulting in increased muscle creatine content. Based on new research, refinements have been made to this strategy and now many athletes consume only one 5 g dose approximately 60 min prior to, or immediately after training (exercise is known to enhance creatine uptake by about 10%) (Bemben and Lamont, 2005). Although responses are quite variable from person to person,

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subjects ingesting creatine average a 2–5 pound greater gain in muscle mass, and 5–15% greater increases in muscle strength and power compared to control (or placebo) subjects (Branch, 2003; Bemben and Lamont, 2005). Creatine supplementation does not appear to enhance endurance-related exercise performance (Bemben and Lamont, 2005).

Research indicates that once muscle stores of creatine are full, they can remain elevated for an additional 4–5 weeks without further supplementation (Snow and Murphy, 2003). Normal healthy adults who continue to use creatine after their muscle stores have reached peak levels may find the additional creatine is converted to creatinine and excreted in the urine (Pline and Smith, 2005). Urinary creatinine levels are commonly used as a marker of kidney function. Individuals who ingest creatine will frequently have elevated creatinine levels—this is normal and represents an increased rate of muscle creatine conversion to creatinine rather than an abnormality of kidney function. The widespread use of this ingredient in dietary supplements suggests a need to evaluate the safety of creatine through quantitative risk assessment.

Most upper safe levels of nutrients and related substances are based on widely applicable risk assessment models used by the US Food and Nutrition Board (FNB) in its Dietary Reference Intakes documents in 1997 and after (Food and Nutrition Board. Institute of Medicine, 1997, 1998a,b, 2000, 2001). The FNB method and reviews are a formalization and extension of the quantitative methods widely used earlier in risk assessment of other substances, and by the food and dietary supplement industries. Because of the systematic, comprehensive and authoritative character of the FNB risk assessment method for nutrients, this approach has gathered widespread support and adoption by others such as the European Commission Scientific Committee on Food (SCF) (European Commission, 2001), the United Kingdom Expert Group on Vitamins and Minerals (EVM) (Food Standards Agency, 2003) and more recently by the Food and Agriculture Organization/World Health Organization project report *A Model for Establishing Upper Levels of Intake for Nutrients and Related Substances* (FAO/WHO, 2006) with some slight modifications. All these reports reflect the concepts and procedures established much earlier for the risk assessment of non-carcinogenic chemicals (National Research Council, 1983).

2. Methods

The safety evaluation method applied to orally administered creatine monohydrate is from the Council for Responsible Nutrition (CRN) *Vitamin and Mineral Safety, 2nd Edition* (Hathcock, 2004), which contains the basic features of the FNB method and also the Observed Safe Level (OSL) modification recently adopted as a Highest Observed Intake (HOI) in the FAO/WHO report.

Overall, this risk analysis was derived from the human clinical trial database through the following major steps:

1. Derive a safe Upper Level of Intake (UL) if the data are appropriate:
   a. Search for data that identify a hazard related to excessive intake.
   b. Assess the dose–response relationship for the identified hazard.
   c. Consider uncertainty and assign an uncertainty factor (UF).
   d. Derive a UL from the No Observed Adverse Intake Level (NOAEL) or Lowest Observed Adverse Effect Level (LOAEL), and the UL = NOAEL ÷ UF.
2. If no data establish adverse effects in humans, the above procedure cannot be used. In these circumstances, identify the highest intake level with sufficient evidence of safety as a value named the OSL by CRN (Hathcock, 2004) and the HOI by FAO/WHO.

We applied the first procedure to the creatine monohydrate human trial data and found no basis for a NOAEL or LOAEL, and thus could not derive a UL. Consequently, we applied the OSL procedure to the clinical trial data, with the results described in the sections below.

No efforts were made in any of the clinical trials to avoid dietary creatine, and therefore the subjects must have been consuming normal dietary levels of this substance. Thus, the OSL value identified from the trials does not require correction for dietary intakes or endogenous synthesis, and the OSL can be identified as a safe Upper Level for Supplements (ULS).

3. Scientific evidence related to safety

Media reports of links between creatine use and muscle strains, muscle cramps, heat intolerance, and other side effects are not supported by the scientific literature. Studies conducted in athletes and military personal indicate a substantial level safety of both short- and long-term creatine use in healthy adults (Poortmans and Francaux, 1999; Robinson et al., 2000; Bennett et al., 2001; Greenwood et al., 2003a,b; Kreider et al., 2003). Concerns about high dose creatine usage causing kidney damage are based solely on a total of two published case reports in which one of the affected individuals was suffering from existing underlying renal disease (Pritchard and Kalra, 1998; Koshy et al., 1999). Both comprehensive literature reviews and expert panels have maintained that there is no conclusive evidence to support the notion that creatine may adversely affect kidney function in healthy individuals (Poortmans and Francaux, 2000; Terjung et al., 2000; Farquhar and Zambraski, 2002; Yoshizumi and Tsourounis, 2004; Pline and Smith, 2005).

3.1. Human studies

There have been nearly 70 peer-reviewed, published human clinical trials involving creatine. Of these, the most relevant randomized, controlled studies that address safety are presented in this review (Table 1). Sample size, dosage and duration, controlling of diet, along with other co-interventions (such as various forms of exercise training), and outcome measures vary considerably between studies. Overall, the literature demonstrates a substantial level of safety with creatine when used in healthy individuals. The primary side effect reported in clinical trials is gastrointestinal upset due to malabsorption of large doses of creatine.

The only form of creatine to be studied in randomized, controlled clinical trials is creatine monohydrate, each gram of which provides 0.879 g of creatine. Therefore, for the purposes of this review, the term "creatine" and accompanying dosages refers to creatine monohydrate. Other

Table 1
Published safety observations for human creatine supplementation

Study	Study population	Dosage and study design	Duration (days)	Key observations	OSL considerations
Kreider et al. (1998) $n = 14$	Div IA college football players	15.75 g/d creatine monohydrate with supervised resistance training; randomized, controlled	28 d	Significant increase in serum creatinine (22.5% to 125 µmol/L[a]); slight but significant increase in liver enzymes; no adverse effects observed	15.75 g, serum creatinine at the upper range, although normal for athletes, but relatively small sample size and short duration argue against use of this study for identification of an OSL
Arciero et al. (2001) $n = 10$	Healthy adult males	20 g/d (5 d) followed by 10 g/d (23 d) creatine monohydrate with supervised resistance training; randomized, controlled	28 d	No outcome measures related to safety reported; no adverse effects reported	10 g in healthy males, but lack of reporting relevant outcome measures and short duration argue against use of this study for identification of an OSL
Watsford et al. (2003) $n = 9$	Healthy adult males	20 g/d (7 d) followed by 10 g/d (21 d) creatine monohydrate; randomized, controlled	28 d	No significant increase in muscular strain or injury following exercise regimen; no adverse effects reported	10 g in healthy males, but lack of relevant outcome measures argue against use of this study for identification of an OSL
Groeneveld et al. (2005) $n = 88$	ALS patients	10 g/d creatine monohydrate; randomized, controlled	310 d	Significant increase in urinary creatine (37-fold to 35.9 mmol/L); no change in serum urea levels or urinary albumin; 3 subjects dropped due to GI side effects; no other differences in adverse effects observed between creatine and placebo	10 g, large sample size and long duration, but study done using disease population argues against use of this study for identification of an OSL
Chrusch et al. (2001) $n = 16$	Elderly males	26 g/d (1 wk d) followed by 6 g/d (11 wk) creatine monohydrate with supervised resistance training; randomized, controlled	12 wk (84 d)	No clinically relevant outcome measures reported; significantly more side effects (gastrointestinal, muscle cramping, muscle pulls) vs. placebo	6 g, moderate duration, but small sample size, population age, and lack of clinically relevant measurements argue against the use of this study for identification of an OSL
Bennett et al. (2001) $n = 8$	Healthy adult males	20 g/d (6 d) followed by 6 g/d (4 wk) creatine monohydrate with supervised military training program; randomized, controlled	34 d	Significant increase in serum (3.6-fold to 1061 µmol/L, 6 d; 2.2-fold to 639 µmol/L, 4 wk) and urinary creatine (400-fold to 2898 µmol/L, 6 d; 115-fold to 840 µmol/L, 4 wk); no significant effect on liver enzymes or core body temperature; no difference in adverse effects observed between creatine and placebo	6 g, slightly higher dose, but small sample size and relatively short duration argue against the use of this study for identification of an OSL
Powers et al. (2003) $n = 16$	Healthy adults	25 g/d (7 d) followed by 5 g/d (21 d) creatine monohydrate with self-maintained resistance training; randomized, controlled	28 d	Significant increases in urinary creatine (32-fold, to 347 µmol/L, 7 d; 24-fold, to 253 µmol/L, 28 d); no change in serum or urinary creatinine levels; no adverse effects observed	5 g in healthy adults, but relatively short duration argues against use of this study for identification of an OSL
Kilduff et al. (2003) $n = 9$	Healthy adult males	22.8 g/d (7 d) followed by 5.7 g/d (21 d) creatine monohydrate with supervised resistance training ; randomized, controlled	28 d	Significant increase in urinary creatinine (2.2-fold, to 3.3 g/24 h); no adverse effects observed	5.7 g in healthy adults, but relatively short duration and small sample size argues against use of this study for identification of an OSL
Derave et al. (2004) $n = 8$	Healthy adults	20 g/d (1 wk) followed by 5 g/d (19 wk) creatine monohydrate; randomized, controlled	20 wk (140 d)	Significant increase in serum creatine (8.4-fold to 210 µmol/L, 1 wk; 4-fold to 100 µmol/L, 10 wk; 3.2-fold to 80 µmol/L, 20 wk); significant 72% increase in urinary creatine (to 7.1 g/24 h); no change in serum urea or urinary creatinine; no adverse effects reported	5 g in healthy adults, substantial duration of exposure; based on the findings of the studies above, this study is chosen to serve as the basis for the human OSL

Op 't Eijnde et al. (2001a) and Hespel et al. (2001) $n=11$	Healthy adults	20 g/d (2 wk) followed by 15 g/d (3 wk) followed by 5 g/d (7 wk) creatine monohydrate with supervised resistance training; randomized, controlled	12 wk (84 d)	No outcome measures related to safety reported; no adverse effects reported	5 g in healthy adults, but lack of relevant outcome measures; supports the OSL selected
Stevenson and Dudley (2001b) $n=12$	Healthy adults	20 g/d (1 wk) followed by 5 g/d (8 wk) creatine monohydrate with self-maintained resistance training; randomized, controlled	9 wk (63 d)	No outcome measures related to safety reported; no adverse effects reported	5 g, moderate duration, but lack of relevant outcome measures; supports the OSL selected
Wilder et al. (2001) $n=8$	Div IA college football players	20 g/d (1 wk) followed by 5 g/d (9 wk) creatine monohydrate with supervised resistance training; randomized, controlled	10 wk (70 d)	No change in urinary creatinine; no adverse effects reported	5 g, supports the OSL selected
Robinson et al. (2000) $n=16$	Healthy adults	20 g/d (5 d) followed by 3 g/d (8 wk) creatine monohydrate with supervised resistance training; randomized, controlled	61 d	Significant increase in serum creatinine (33% to 90 μmol/L[a]); no change in serum urea and other hematological indices; no adverse effects observed	3 g, serum creatinine within the normal range; supports OSL selected
van Loon et al. (2003) $n=10$	Healthy adult males	20 g/d (5 d) followed by 2 g/d (6 wk) creatine monohydrate; randomized, controlled	47 d	No outcome measures related to safety reported; no adverse effects reported	2 g, moderate duration, but lack of relevant outcome measures; supports the OSL selected
Derave et al. (2003) $n=22$	Healthy adults	15 g/d (2 wk) followed by 2.5 g/d (6 wk) creatine monohydrate with supervised resistance training; randomized, controlled	8 wk (56 d)	Significant increase in total muscle creatine content; no adverse effects reported	2.5 g, supports the OSL selected
Eijnde et al. (2003) $n=23$, 10*	Healthy older adult men	5 g/d creatine monohydrate; with supervised cardiovascular and resistance training; randomized, controlled	6 mo, 1 yr* (153 d, 365 d*)	Significant increase in urinary creatine (4.6-fold to 3.46 g/24 h); significant increase in serum creatine (10% to 95 μmol/L); no difference in adverse effects observed between creatine and placebo	5 g, supports the OSL selected
Verbessem et al. (2003) $n=26$	Huntington's disease patients	5 g/d creatine monohydrate; randomized, controlled	1 yr (365 d)	Significant increase in urinary creatine, serum creatinine (data not provided); no change in serum urea; no difference in creatinine clearance vs. placebo; no adverse effects reported	5 g, moderate sample size and long duration, but study done using disease population; supports the OSL selected
Escolar et al. (2005) $n=15$	Pre-adolescent dystrophic patients	5 g/d creatine monohydrate; randomized, controlled	6 mo (180 d)	No clinically relevant outcome measures reported; no difference in adverse effects observed between creatine and placebo	5 g, small sample size and long duration, but study done using disease population; supports the OSL selected
Tarnopolsky et al. (2004b) $n=30$	Pre-adolescent dystrophic patients	5 g/d creatine monohydrate; randomized, controlled, crossover	4 mo (120 d)	No change in serum or urinary creatinine or creatinine clearance; no adverse effects reported	5 g, moderate sample size and long duration, but study done using disease population; supports the OSL selected
Tarnopolsky et al. (2004a) $n=34$	Adult dystrophic patients	5 g/d creatine monohydrate; randomized, controlled, crossover	4 mo (120 d)	Significant increase in serum creatinine; no change in creatinine clearance; no adverse effects reported	5 g, moderate sample size and long duration, but study done using disease population; supports the OSL selected
Walter et al. (2000) $n=32$	Dystrophic patients	5–10 g/d creatine monohydrate; randomized, controlled, crossover	8 wk (56 d)	No changes in any clinical chemistry endpoints reported; no adverse effects observed	5, 10 g, moderate sample size and duration, but study done using disease population; supports the OSL selected

(continued on next page)

Table 1 (*continued*)

Study	Study population	Dosage and study design	Duration (days)	Key observations	OSL considerations
Louis et al. (2003a) $n = 15$	Pre-adolescent and adolescent dystrophic patients	3 g/d creatine monohydrate; randomized, controlled, crossover	3 mo (90 d)	Significant increase in serum (2.8-fold to 331 μmol/L) and urinary (2.8-fold to 1.7 g/24 h) creatine; no change in serum or urinary creatinine or urinary albumin; no change in liver enzymes; no adverse effects observed	3 g, reasonable duration and all relevant endpoints assessed, but study done using disease population; supports the OSL selected

[a] Normal range for serum creatinine = 50–115 μmol.

criteria for study inclusion were study duration (more than one week), and studies had to be randomized, placebo-controlled intervention trials. Studies that were uncontrolled and unblinded, observational, those investigating acute bioavailability, pharmacokinetics or postprandial responses from single bolus doses were excluded from this analysis, and are used solely as supportive information.

A large portion of the clinical trials conducted with creatine using healthy adults have the used a "loading phase" (typically 20 g/d creatine, for 3 d to 1 wk in duration), followed by a "maintenance phase" (typically 5–6 g/d, varying duration). The rationale being that the large dose for a short period at the outset of supplementation may help facilitate creatine uptake by skeletal muscle (Bemben and Lamont, 2005). For the purposes of this review, rather than being used as the basis for short-term recommendations, loading doses are used as support for recommendations for a lower maintenance dose deemed safe for long-term use.

Several human studies have focused specifically on the safety issues of creatine supplementation. In 2003, Kreider et al. and Greenwood et al. published two separate reports on an open label study involving creatine supplementation in Division IA college football players. Both reports concluded that creatine doses ranging from 5 to 15 g/day for 21 months did not increase the incidence of cramping or injury, and had no adverse effects on hepatic or renal function (Greenwood et al., 2003a,b; Kreider et al., 2003). Other uncontrolled trials have reported similar findings in athletes with respect to renal function and thermoregulation (Poortmans and Francaux, 1999; Schilling et al., 2001; Mayhew et al., 2002; Potteiger et al., 2002; Rosene et al., 2004). Although not randomized, double-blind, controlled trials, these reports help to provide confidence in the remaining body of research demonstrating the safety of creatine.

Americans consume approximately one gram of creatine per day from the diet, with the equivalent amount produced endogenously (Balsom et al., 1994), suggesting that the doses used in the reviewed trials are 3- to 12-fold higher and are adequate to assess safety of supplementation. The absence of any pattern of adverse effects related to creatine supplementation in any of the published human trials provides support for a high level of confidence in the safety of this compound.

3.2. *Animal and in vitro studies*

Although many studies on creatine using animal models have been published, few have specifically focused on safety and toxicity. Of these, only one reported adverse effects using a rat model of renal cystic disease (Edmunds et al., 2001). Using a creatine loading dose, followed by a maintenance dose (human equivalent of 20 g/d, 1 wk followed by 5 g/d, 5 wk) analogous to that used in human trials, Edmunds et al. reported that this regimen contributed to reduced renal function (increased kidney weight, increased serum urea level, lower creatinine clearances) in Sprague–Dawley rats with existing cystic kidney disease. These results are in contrast with a study published by Taes et al. (2003), who reported that creatine supplementation does not affect kidney function in rats with pre-existing renal failure. Researchers provided sham-operated and partially nephrectomized (effectively inducing renal failure) rats either a control diet, or creatine diet (providing 0.9 g/kg body weight creatine/d; equivalent to approximately 50 g/d in 60 kg adult human) for four weeks (Taes et al., 2003). There was no effect of creatine supplementation on inulin or creatinine clearance rates, urinary protein excretion or urea clearance.

A histological assessment of the effect of up to one year of creatine supplementation revealed that 0.05 g/kg bw/d in mice resulted in inflammation of the liver (no other organs affected), while a supraphysiologic dose in rats (2% creatine diet) caused no pathological effects in any of the organs analyzed (Tarnopolsky et al., 2003). These results suggest that the effects of creatine are species specific and that the rat model more closely represents humans.

Other creatine studies conducted using animal models (mice, rats, guinea pigs, dogs; doses ranging from 0.05 to 2 g/kg body weight/d for between 2 and 8 wk) examining serum, muscle and organ concentrations, while not focused on safety or toxicity, have not reported any adverse effects (Lowe et al., 1998; Ipsiroglu et al., 2001; Ju et al., 2005). These provide additional support that creatine supplementation at doses analogous to or higher than those used in humans do not cause adverse effects in most animals under normal conditions.

4. Human NOAEL or OSL (HOI)

Research on creatine safety and toxicity has focused primarily on its effect on renal function. This stems from the knowledge that excess creatine is eliminated from the body via glomerular filtration in the kidney either as creatine or its metabolite, creatinine (Ropero-Miller et al., 2000). Two human case reports (Pritchard and Kalra, 1998; Koshy et al., 1999) and a single study in rats with renal disease (Edmunds et al., 2001) have also fueled concerns about creatine's affect on the kidney. In clinical practice, several marker compounds are used to assess renal function, including serum creatinine and urea levels, urinary albumin and inulin clearance (Farquhar and Zambraski, 2002). Elevated serum levels of creatinine well beyond the normal range (50–115 μmol/L) can be indicative of reduced clearance rates, and along with increased urinary albumin denote compromised renal function. Both controlled and uncontrolled clinical trials involving creatine supplementation have demonstrated elevations in one or more of the following markers: serum creatine and creatinine, and urinary creatine and creatinine. In all cases these elevations have been within the normal range.

creatinine
mg/dL
0.57
0.57-1.30

Although not reviewed in detail here, published clinical trials lasting one week or less, which comprise approximately half of the total number of clinical trials conducted on creatine, are generally supportive of the longer-term trials. Regardless of dosage (ranging from 5 to 30 g/d) or study population, none of the short-term trials (ranging from 3 to 7 d), report any adverse effects, and where measured, all relevant endpoints are within the normal range (Gordon et al., 1995; Jacobs et al., 1997; Odland et al., 1997; Poortmans et al., 1997; Volek et al., 1997; Maganaris and Maughan, 1998; Smith et al., 1998a,b; Bellinger et al., 2000; Mihic et al., 2000; Nelson et al., 2000; Burke et al., 2001; Op 't Eijnde and Hespel, 2001; Op 't Eijnde et al., 2001b; Stevenson and Dudley, 2001a; Volek et al., 2001; Cottrell et al., 2002; Cox et al., 2002; Jacobs et al., 2002; Ziegenfuss et al., 2002; Louis et al., 2003b,c; Anomasiri et al., 2004; Eckerson et al., 2004; Ostojic, 2004; Mendel et al., 2005; Theodorou et al., 2005). Aside from the case reports, none of the clinical studies involving healthy adults or those using neuromuscular disease patients have found reduced renal clearance rates (as indicated by above normal elevations in serum creatinine or urea), or increased urinary albumin. Other alleged adverse effects of creatine supplementation, such as cramping and increased core body temperature have not been observed in any of the controlled or uncontrolled human trials.

4.1. Human NOAEL

Given that none of the clinical trials found a clear adverse effect related to creatine administration, there is, by definition, no basis for identifying a LOAEL. In the absence of a LOAEL, a NOAEL is not usually set. Without either of these two values the establishment of a UL usually is not set (Food and Nutrition Board. Institute of Medicine, 1998b).

4.2. Human OSL

Published relevant human clinical trials involved creatine doses of up to 26 g/d ("loading" phase) and 6 g/d ("maintenance" phase) (Chrusch et al., 2001). All human studies reviewed were double-blind, randomized, controlled trials (Table 1). A series of non-randomized, open-label clinical trials has also been published. The dosages involved in these studies ranges from 1 to 30 g/d for up to five years, the results of which are consistent with respect to safety, showing no observed or reported adverse effects (Poortmans and Francaux, 1999; Schilling et al., 2001; Mayhew et al., 2002; Potteiger et al., 2002; Greenwood et al., 2003a; Greenwood et al., 2003b; Kreider et al., 2003; Rosene et al., 2004).

Of the randomized, controlled clinical trials reviewed in this report, two studies reported gastrointestinal side effects (Chrusch et al., 2001; Groeneveld et al., 2005). One study involved elderly men and described the side effects as few and minor (Chrusch et al., 2001). The other reported that a few subjects taking creatine left the study due to gastrointestinal upset. The same study reported no

$$\frac{50\ \mu mol}{L}\bigg|\frac{113.12\ \mu g}{1\ \mu mol}\bigg|\frac{mg}{1000\ \mu g}\bigg|\frac{L}{10\ dL} = 0.57\ mg/dL$$

1.3 mg/dL

significant difference overall in side effects between creatine and placebo (Groeneveld et al., 2005). The remaining studies report a complete absence of side effects, do not address the issue of side effects, or report no difference in the incidence of side effects between creatine and placebo. With respect to clinically relevant markers, none of the studies reviewed showed clinically relevant changes in serum creatinine or urea, urinary albumin, or liver enzymes. A total of two studies using healthy adult subjects reported statistically significant increases in serum creatinine. Robinson et al. (2000) reported a 33% increase in serum creatinine (to 90 μmol/L) in healthy adults who received 20 g/d creatine (5 d) followed by 3 g/d creatine (8 wk) combined with a supervised resistance training program. Kreider et al. (1998) reported a 22.5% increase in serum creatinine (to 125 μmol/L) in Division IA college football players who received 15.75 g/d creatine along with a supervised resistance training program for 28 d. Although elevated, these values are still recognized as being within the normal range for serum creatinine and likely are reflective of an increased conversion of creatine to creatinine within the body and not renal dysfunction (Farquhar and Zambraski, 2002).

4.3. 15.75 g/d

Kreider et al. reported no adverse effects in Div. IA college football players who ingested 15.75 g/d creatine for 28 d (Kreider et al., 1998), but did observe a significant increase in serum creatinine levels (to 125 μmol/L). The interpretation of these results is confounded by the fact that these athletes were also undergoing an intense exercise training regimen. While the serum creatinine level is still within a normal range for this population, the relatively small sample size ($n = 14$) and short duration argue against use of this study for identification of an OSL.

4.4. 10 g/d

Three studies implement the use of a 10 g/d maintenance dose. Arciero et al. (2001) and Watsford et al. (2003) use very similar study designs involving healthy adult male subjects and a 20 g/d (5 and 7 d, respectively) and a 10 g/d maintenance dose (up to d 28). While neither study reported any adverse effects, they also failed to measure or report any clinically relevant safety outcomes (such as serum or urinary markers). This fact, combined with the relatively short duration, argue against use of this study for identification of an OSL. The final study using a 10 g/d dose involved amyotrophic lateral sclerosis (ALS) patients, who were studied for a total of 310 d (Groeneveld et al., 2005). Although some minor gastrointestinal side effects were observed, there was no change in serum urea or urinary albumin levels. The long duration and modest sample size ($n = 88$) provide confidence in the safety of this dose, however, the diseased nature of the study population argue against use of these results for identification of an OSL.

4.5. 6 g/d

The gastrointestinal side effects reported by Chrusch were described as few and minor (Chrusch et al., 2001). This study utilized the largest dose of creatine for the "loading phase" (26 g/d) in healthy subjects and a dose of 6 g/d in the "maintenance" phase, but failed to measure or report any clinically relevant safety outcomes. Although it was of moderate duration (84 d), the small sample size, population age (elderly men), and lack of clinically relevant measurements argue against the use of this study for identification of an OSL. Bennett et al. (2001) implemented a very similar dosing regimen (20 g/d, followed by 6 g/d) and found no adverse effects, including no clinically relevant changes in safety markers. Powers et al. (2003) observed no increase in serum or urinary creatinine after a dosing regimen of 25 g/d (7 d) followed by 5 g/d (21 d). Kilduff et al. (2003) (22.8 g/d, 7 d, followed by 5.7 g/d, 21 d) reported no adverse effects and only a normal rise in urinary creatinine. However, the relatively small sample sizes and short duration employed in these studies argue against their use for identification of an OSL.

4.6. 5 g/d

Although a relatively small sample size ($n = 8$), Derave et al. (2004) exposed subjects to a relatively large loading dose (20 g/d, 7 d) followed by 5 g/d for 19 wk, for a total exposure of 140 d in healthy adults. The study also showed no significant increase in either serum creatinine or urea. Therefore, given the substantial duration of exposure and the findings of the studies including creatine doses at, above and below this level, this study is chosen to serve as the basis for the human OSL of 5 g/d.

The remainder of the relevant studies provided in Table 1 employed creatine maintenance doses of 5 g/d or lower for durations of up to one year in varying populations serve to support and provide confidence in the selected OSL (Robinson et al., 2000; Walter et al., 2000; Hespel et al., 2001; Op 't Eijnde et al., 2001a; Stevenson and Dudley, 2001b; Wilder et al., 2001; Derave et al., 2003; Eijnde et al., 2003; Louis et al., 2003a; van Loon et al., 2003; Verbessem et al., 2003; Tarnopolsky et al., 2004a,b; Escolar et al., 2005). In all of these studies, there were either no adverse effects observed by the investigators or none reported. One study (Robinson et al., 2000) reported a significant increase in serum creatinine, the level of which (90 μmol/L) is still well within the normal range. The quantities of creatine involved in these trials are supplemental amounts well above the estimated amount consumed in foods consumed in the U.S. (1 g/d (Balsom et al., 1994)). Therefore, this risk assessment represents a direct approach to an ULS. No correction is needed for the creatine present in the U.S. food supply.

4.7. Uncertainty evaluation

The study chosen as the basis for the human OSL (Derave et al., 2004), on its own merit alone, would necessitate the application of a relatively high UF, due to its small sample size ($n = 8$). However, there is a collection of other randomized controlled trials conducted in healthy adults using creatine doses at, above and below 5 g/d, all demonstrating and/or reporting no adverse effects. These serve as support for and provide confidence in the results of the study by Derave et al.

NOAEL and LOAEL: >10 g/d
OSL: 5 g/d
ULS: Because the 5 g/d dose was administered to subjects eating normal diets, no correction of the OSL for dietary intake is needed, therefore OSL = ULS = 5 g/d

5. Conclusions

The body of evidence supporting a beneficial effect of creatine supplementation performance outcomes continues to grow. The increased availability and appearance of this ingredient in dietary supplement products, along with continuing accusations of potential adverse effects, warranted the present risk assessment. Application of risk assessment methodology to the available published human clinical trial data involving creatine supports a high level of confidence in this ingredient with respect to its safe use in dietary supplements. The absence of a well-defined critical effect precludes the selection of a NOAEL, and therefore required use of the observed safe level (OSL) or highest observed intake (HOI) approach established by FAO/WHO to conduct this risk assessment. Evidence from well-designed randomized, controlled human clinical trials indicates that the Upper Level for Supplements (ULS) for creatine is 5 g per day.

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Review Section

ASSESSMENT OF THE RISK OF FORMATION OF CARCINOGENIC *N*-NITROSO COMPOUNDS FROM DIETARY PRECURSORS IN THE STOMACH

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Summary—A literature review has shown that the daily intakes of various *N*-nitroso-precursor classes in a typical European diet span five orders of magnitude. Amides in the form of protein, and guanidines in the form of creatine and creatinine, are the nitrosatable groups found most abundantly in the diet, approaching levels of 100 g/day and 1 g/day, respectively. Approximately 100 mg of primary amines and amino acids are consumed daily, whereas aryl amines, secondary amines and ureas appear to lie in the 1–10 mg range. The ease of nitrosation of each precursor was estimated, the reactivities being found to span seven orders of magnitude, with ureas at the top and amines at the bottom of the scale. From this information and an assessment of the carcinogenicity of the resulting *N*-nitroso derivatives, the potential health risk due to gastric *in vivo* nitrosation was calculated. The combined effects of these risk variables were analysed using a simple mathematical model: Risk = [daily intake of precursor] × [gastric concentration of nitrite]^n × [nitrosatability rate constant] × [carcinogenicity of derivative]. The risk estimates for the various dietary components spanned nine orders of magnitude. Dietary ureas and aromatic amines combined with a high nitrite burden could pose as great a risk as the intake of preformed dimethylnitrosamine in the diet. In contrast, the risk posed by the *in vivo* nitrosation of primary and secondary amines is probably negligibly small. The risk contribution by amides (including protein), guanidines and primary amino acids is intermediate between these two extremes. Thus three priorities for future work are a comprehensive study of the sources and levels of arylamines and ureas in the diet, determination of the carcinogenic potencies of key nitrosated products to replace the necessarily vague categories used so far, and the development of short-term *in situ* tests for studying the alkylating power or genotoxicity of *N*-nitroso compounds too unstable for inclusion in long-term studies.

Introduction

The consumption of nitrite and dietary nitrogen-containing compounds might be an aetiological factor in the development of some cancers, particularly of the gastro-intestinal tract, a possibility reviewed by Mirvish (1983), Sander (1971) and Tannenbaum *et al.* (1977). Under the acidic conditions found in the stomach, these compounds form nitrosamines and nitrosamides (Meier-Bratschi *et al.* 1983; Sander, 1971). The *N*-nitroso compounds (NOCs) produce reactive electrophiles which can bind to cellular nucleophiles (Fig. 1), among them DNA (Druckrey *et al.* 1967). The carcinogenic potency of NOCs is thought to rest primarily on their ability to form promutagenic DNA adducts (Lutz, 1979; Magee, 1977; Magee & Barnes, 1967; Magee & Farber, 1962).

The risk associated with the intake of *N*-nitroso precursors is a function of three variables: (1) the amount of precursor and nitrite ingested, (2) the rate of *in vivo* nitrosation and (3) the carcinogenic potency of the resulting NOC. Studies related to the first variable have concentrated mainly on the consumption of nitrite in the diet and have been reviewed by Ellen & Schuller (1983). Sodium nitrite, used primarily in preserving meats, is the best-known source of dietary nitrite. In Europe and North America the average daily intake of sodium nitrite lies around 4 mg/person. Nitrates may also play a role, as they can be reduced to nitrite in the human oral cavity. Nitrates are found in food in considerably higher quantities than nitrites; green leafy vegetables are particularly rich in nitrate (Ellen & Schuller, 1983). The average daily consumption of nitrate ions lies at approximately 100 mg/person/day in Western countries. According to Stephany & Schuller (1980), about 6% of ingested nitrate is reduced endogenously to nitrite; the 6 mg derived from nitrate would thus raise the daily nitrite burden to 10 mg/person.

Abbreviations: DMN = dimethylnitrosamine; MNNG = *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; NOC = *N*-nitroso compound; OPI = oncogenic potency index.

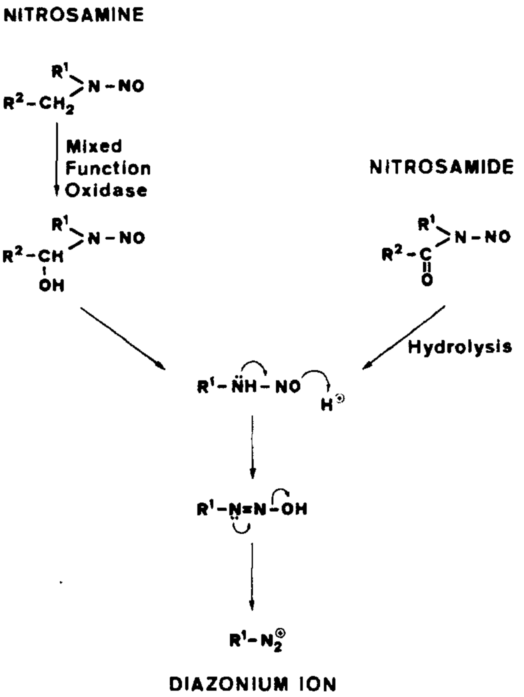

Fig. 1. Formation of electrophilic products by spontaneous or enzymatic degradation of *N*-nitroso compounds.

Drugs and other manufactured compounds contain groups that may react with nitrite to form NOCs (Coulston & Dunne, 1980; Lijinsky, 1981a). The contribution of amines and amides found in the natural diet has received less attention, with the notable exception of work by Mirvish (1971 & 1972), Möhler *et al.* (1972) and Walker (1981), although these compounds constitute an unavoidable source of *N*-nitroso precursors for the general population.

The measurement of *in vivo* nitrosation (the second variable) has been carried out using a variety of short-term tests, notably, examination of stomach contents after feeding nitrite and the test substance to rats (Lijinsky, 1981a), measurement of NOCs in human body fluids after ingestion of proline and nitrate (Ohshima & Bartsch, 1981), of amidopyrine (Spiegelhalder & Preussmann, 1984) or of piperazine (Bellander *et al.* 1984), isolation of DNA alkylated by metabolites or breakdown products of NOCs (Huber & Lutz, 1984; Meier-Bratschi *et al.*, 1983) or estimation of *in vivo* nitrosation through long-term carcinogenicity studies (Sander, 1971). Compounds tested include manufactured compounds, such as pesticides and drugs, and some food components. However, a systematic look at the *in vivo* nitrosatability of natural dietary *N*-nitroso precursors has not yet been undertaken. The kinetic work done to date suggests, however, that the rate of *in vivo* nitrosation correlates reasonably well with *in vitro* kinetic studies (Meier-Bratschi *et al.* 1983; Ohshima *et al.* 1983). Thus, estimates of the relative ease of nitrosation of the various classes of compound could be made on the basis of extensive *in vitro* kinetic work carried out by Mirvish (1975).

With respect to the third variable, Druckrey *et al.* (1967) have shown that NOCs, as a class, are extremely potent carcinogens. More recently, several studies have attempted to quantify and compare the strengths of strong carcinogens on the basis of an evaluation of long-term carcinogenicity data. Meselson & Russell (1977) developed a mathematical model describing the daily dose and duration of treatment necessary to induce a given number of tumours over the lifetime of rodents. Parodi *et al.* (1982) adapted the model, naming it the oncogenic potency index (OPI), and calculated the potencies of the best studied NOCs. No potency estimates, however, have as yet been calculated for many of the *N*-nitroso derivatives that could be formed *in vivo*.

In this review, the most widespread classes of naturally occurring NOC precursors are evaluated in detail with respect to each risk criterion: the most important dietary precursors of NOCs are listed; information is provided on the *in vitro* or *in vivo* nitrosation rate and an indication of the carcinogenic potency of the corresponding NOC is given. Furthermore, the combined effects of these risk variables are analysed using a simple mathematical model:

$$\begin{aligned}\text{Risk} = {} & [\text{daily intake of precursor}] \\ & \times [\text{gastric concn of nitrite}]^{n} \\ & \times [\text{nitrosatability rate constant}] \\ & \times [\text{carcinogenicity of derivative}] \quad \ldots\ldots (1)\end{aligned}$$

where the exponent 'n' equals 2 for amine nitrosation and 1 for amide-type nitrosation.

The quantitative risk assessments generated with this model are then used to identify the precursors (and precursor classes) and physiological and nutritional states where *in vivo* nitrosation poses the largest health risk.

Precursors of NOCs in the diet

Figure 2 lists the types of compounds that can form *N*-nitroso derivatives. Widely occurring dietary nitrosatable compounds are amines, amides, guanidines and ureas. Hydrazine derivatives could not be included in the study, first because no information on their kinetics of nitrosation was available and secondly because many hydrazine compounds are known to possess mutagenic and carcinogenic activity in their own right, without requiring prior nitrosation (Kimball, 1977; Parodi *et al.* 1981). The remaining compounds, while synthesized industrially, are not known to be natural dietary constituents and were therefore not included in this risk assessment. The study did not examine precursors of C- or S-nitroso compounds.

Primary amines

A wide variety of primary amines is found in foods, particularly in those that are produced by or subjected to microbial fermentation, such as cheese, meat and fish (Walker, 1981). Methylamine is the most widespread member of this class of compounds, and has been found in vegetables, stimulants, grains, cheeses, fish, pickles and meat (Neurath, 1977). For a complete list of these foods, see Appendix I.

Other amines arising from decarboxylated amino acids are found in substantial amounts, particularly

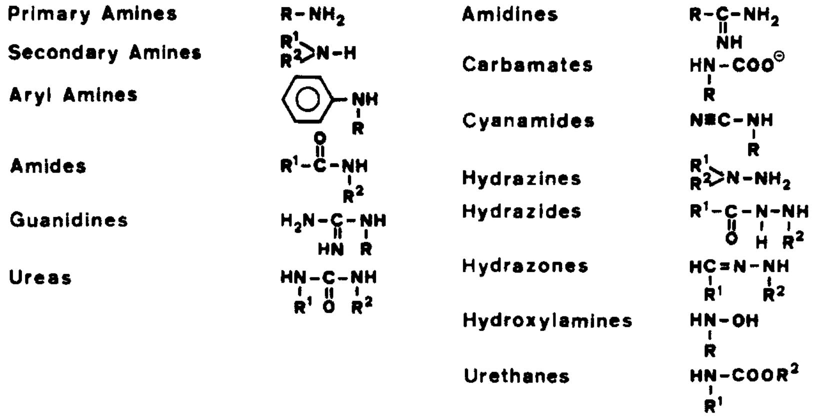

Fig. 2. Names and general formulae of *N*-nitroso-compound precursors.

in meat or fish that has begun to spoil, and in mature cheeses. The most important amines of this type are tyramine, cadaverine, putrescine, tryptamine and histamine (Belitz & Schormüller, 1965; Gangolli, 1981). Ethylamine, isopentylamine and ethanolamine are also found, although to a lesser extent than methylamine. Furthermore, the polyamines spermidine and spermine, along with serotonin, dopamine and sphingosine, have been reported in various types of food. Benzylamine and *n*-propyl- and *n*-pentylamine are minor components of vegetables (Neurath, 1977). A complete list of dietary sources of primary amines is found in Appendix I.

From the amounts of the various food items consumed per person and year in Switzerland, the average daily intake of each primary amine was estimated. Food consumption tables were drawn from *Produktion und Verbrauch von Nahrungsmitteln in der Schweiz 1969/70 bis 1980* (Schweizerisches Bauernsekretariat, 1983) using the 1980 figures. From the calculations compiled in Table 1, it is apparent that the so-called 'endogenous' amines and the polyamines spermidine, tyramine, cadaverine, putrescine and tryptamine are the major dietary amines.

Table 1. Approximate daily intake of primary amines and amino acids

Compound	Daily intake (mg/person)
Amines*	
Spermidine	35
Tyramine	21
Cadaverine	15
Putrescine	15
Tryptamine	9
Spermine	5
Methylamine	3
n-Propylamine	2
Histamine	2
Hexylamine	2
Serotonin	1
Ethanolamine	0.7
Ethylamine	0.6
Benzylamine	0.5
n-Butylamine	0.4
n-Pentylamine	0.3
Phenylethylamine	0.3
Isopentylamine	0.2
Dopamine	0.2
n-Dodecylamine	0.03
Isopropylamine	0.01
Isobutylamine	0.005
Amino acids†	
Glutamic acid	>3.2
Glycine	>1.3
Alanine	>0.4
Asparagine	>0.3
Serine	>0.3

*Intake values based on typical Swiss diet.
†Intake values based on amino acid content of milk.

Amino acids (see Appendix I) form an important subclass of primary amines. Meat contains relatively high concentrations of free aspartic acid, glutamic acid, alanine, glycine, serine and glutamine (Grau, 1968). Milk is also rich in all but the last of these particular amino acids. Of primary importance among the amino acids found in grains are glutamic acid, arginine and leucine (Rohrlich & Thomas, 1967). Cheese and eggs are also likely to contain fair amounts of free amino acids. In a comprehensive study on the free amino acid composition of mushrooms, Oka *et al.* (1981) found glutamic acid and alanine in the highest amounts (both 1.3 g/kg), followed by arginine (0.9 g/kg). Using the levels of free amino acids in milk as given by Kirchmeier (1968), a minimum daily intake of the major amino acids was calculated. The results, in Table 1, indicate that glutamic acid and glycine are consumed to the largest extent.

Secondary amines

Here again, the low-molecular-weight analogues, dimethyl-, methylethyl- and diethylamine, are the most widespread in foods (see Appendix I). They have been found in all the food classes examined. Also of importance are pyrrolidine, piperidine, *N*-methylbenzylamine, *N*-methylphenethylamine, chavicine and dipropyl- and dibutylamine (Neurath, 1977). Neurath (1977) found that the concentration of individual secondary amines rarely exceeded 10 ppm in vegetables, grains, stimulants, herring and four types of cheese.

The average daily intake of the major secondary amines was also calculated. As can be seen from Table 2, the quantities are, with the exception of dimethylamine, significantly smaller than those for the major primary amines.

Table 2. Approximate daily intake of secondary amines, secondary amino acids and aromatic amines

Compound	Daily intake* (mg/person)
Amines	
Dimethylamine	1.7
Pyrrolidine	0.6
N-Methylbenzylamine	0.6
N-Methylphenethylamine	0.3
Methylethylamine	0.3
Diethylamine	0.1
Piperidine	0.06
Pyrroline	0.03
Amino acids	
Proline	0.47
Sarcosine	1†
Aromatic amines	
N-Methylaniline	1.6
Aniline	1.0
Toluidines	0.2

*Values based on typical Swiss diet.
†Estimate for use in risk calculation (see text).

The secondary amino acids proline, 3-hydroxyproline and sarcosine are widely found. Proline, bound in proteins, is an important component of grains (Rohrlich & Thomas, 1967), and free proline has been reported at levels of 1.0 g/kg in mushrooms (Oka *et al.* 1981) and 1.5 ppm in milk (Kirchmeier, 1968). This last value would give a minimum daily intake of 0.47 mg proline per person (Table 2). Sarcosine has been reported in lobster and cartilaginous fish (Belitz & Schormüller, 1965).

Arylamines

The only two arylamines that seem to be widespread are aniline and *N*-methylaniline. According to Neurath (1977), aniline is found primarily in rapeseed cake (120 ppm) and carrots (31 ppm), and *N*-methylaniline in cheese (38 ppm). Other sources are listed in Appendix I and the daily intakes of these compounds, and of toluidines, are given in Table 2.

Amides

In the form of proteins, amides are consumed in large quantities in meat, milk, eggs, fish, poultry and grains. The daily intake amounts to 92 g/day (Table 3; Schweizerisches Bauernsekretariat, 1983). These are broken down to oligopeptides in the stomach by pepsin. Of the low-molecular-weight amides, asparagine and glutamine—as amino acids—are the most important (Appendix I). Carrot juice and asparagus are particularly rich in asparagine. Furthermore, glutamic acid, the most abundant amino acid, is easily converted by ring closure to pyrrolidone carboxylic acid, its amide. Glutathione is widely found in mammalian liver and muscle. Carnosine and anserine are also found in the muscles of mammals, fish and poultry, the former at values fluctuating between 90 and 4600 mg/kg meat (Belitz & Schormüller, 1965).

Table 3. Preliminary estimates of daily amide, guanidine and urea intakes

Compound	Daily intake* (mg/person)
Amides	
Protein	92,000
Carnosine	2000
Guanidines	
Creatine	800
Creatinine	300
Agmatine	0.7
Methylguanidine	0.2
Ureas	
Methylurea	1
N-Carbamoylputrescine	1†
Citrulline	1†

*Values based on typical Swiss diet.
†Estimate for use in risk calculation (see text).

Guanidines

Creatine and creatinine are important constituents of meat, comprising together 2.5% of the total protein (Grau, 1969). Arginine is an endogenous guanidine found in the urea cycle. Methylguanidine has been reported in beef and fish in concentrations up to 0.2% (Mirvish, 1972). Fresh abalone contains agmatine, the decarboxylation product of arginine (Kawabata *et al.* 1978; Appendix I) while arginine itself is found in mushrooms (0.9 g/kg; Oka *et al.* 1981) and in soy sauce. Canavanine (found in beans), arcaine and glycocyamine are the other major guanidines that have been reported in foods (Mirvish, 1972). From the few quantitative data on guanidines in food, crude estimates of the daily intake per person have been calculated (Table 3).

Ureas

Citrulline is the most important of the naturally occurring urea compounds (listed in Appendix I) because of its endogenous role as a component of the urea cycle. Citrulline has also been found in watermelons, green peppers and soya sauce. Albizziin (reported in plants of the Mimosa family) and hydantoic acid are also constituents of foods. Ureas are important as intermediates in pyrimidine and purine metabolism and are also formed by enzymatic carbamoylation of amines and de-imidation of guanidines. Of these compounds, *N*-carbamoylputrescine, allantoic acid, hydantoic acid and *N*-carbamoylaspartic acid are the best known. The first three ureas are found in plants, and all but hydantoic acid have been detected in the urine of animals or man (Mirvish, 1972).

Cooking or frying creatine-containing foods appears to be a source of methylurea, produced by the breakdown of the intermediate creatinine. Mirvish *et al.* (1980b) have recently reported finding 25 ppm methylurea both in a Japanese fish product and in fried bacon. The same paper reports the finding of longer chain alkylureas in foods. Furthermore, Japanese papers have recently started to suggest that urea compounds may be important components of processed or cooked sea foods (Kodama *et al.* 1982). A rough estimate of methylurea intake was calculated from these data to be 1 mg/day (Table 3).

Comment

The intake of the various *N*-nitroso precursors spans five orders of magnitude. Amides, in the form of protein, and guanidines, in the form of creatine and creatinine, are the nitrosatable groups found most abundantly in the diet, approaching levels of

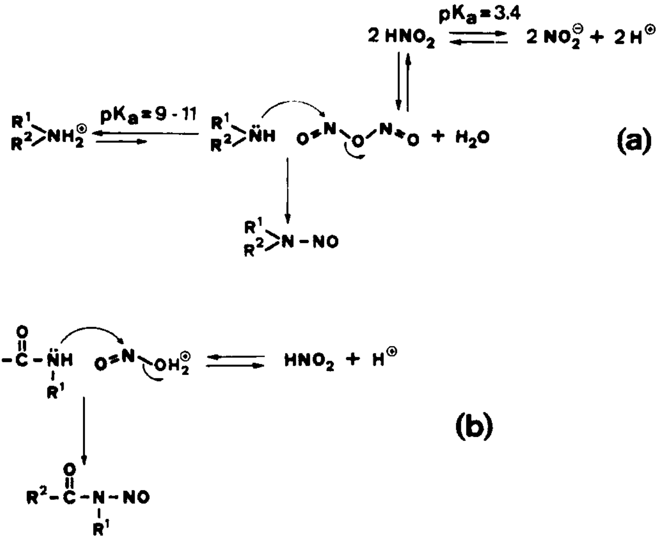
pKa=3.4
2 HNO2 ⇌ 2 NO2⁻ + 2 H⁺
R¹R²NH2⁺ pKa=9-11 R¹R²NH O=N–O–N=O + H2O
(a)
R¹R²N-NO
R²-C(=O)-NH-R¹ O=N-OH2⁺ ⇌ HNO2 + H⁺
(b)
R²-C(=O)-N(R¹)-NO

Fig. 3. Mechanisms of nitrosation of (a) amines and (b) amides.

100 and 1 g/day, respectively. Approximately 100 mg primary amines and free amino acids are consumed daily, whereas arylamines, secondary amines and ureas appear to lie in the 1–10 mg range. It should be noted, however, that sensitive analytical methods for the quantitative detection of non-volatile amides, guanidines and ureas in foods are still in the developmental stages. For this reason, daily intake estimates for these types of compounds should be regarded as preliminary, representing only the minimum ingested values.

Where no quantitative data on occurrence were available, an arbitrary minimum daily intake of 1 mg/person was assigned for the purpose of calculating a risk estimate. Such compounds are distinguished with a footnote in Tables 2 and 3.

Nitrosatability

Kinetic experiments on the *in vivo* nitrosation of dietary precursors in the human stomach have, for ethical reasons, only been carried out using proline and nitrate, since nitrosoproline is considered to be a non-carcinogen (Ohshima & Bartsch, 1981). The same authors also studied the kinetics of the *in vivo* nitrosation of proline, sarcosine and hydroxyproline by nitrite in rats (Ohshima *et al.* 1982 & 1983). They were able to show that the kinetic equation found *in vitro* (see below) was applicable to nitrosation in the rat stomach and was also compatible with the human data. Furthermore, they found that the relative nitrosation reactivity of the three amino acids *in vivo* matched the relative rates *in vitro* published by Mirvish (1975). Finally, the nitrosation yields of proline *in vivo* and *in vitro* at 37°C differed only by a factor of 4 (*in vitro* > *in vivo*). Iqbal *et al.* (1980) carried out *in vivo* nitrosation studies in mice using morpholine or dimethylamine plus nitrite and concluded that the *in vivo* and *in vitro* nitrosation rates corresponded well. These experiments suggest that, in the absence of *in vivo* data, knowledge of the *in vitro* nitrosation rate of the various precursors would provide a reasonable basis for predicting the relative nitrosatabilities *in vivo*.

Unfortunately, nitrosation rate constants have not been measured for most of the naturally occurring nitrosatable compounds, and the available *in vitro* studies either were not carried out under simulated gastric juice conditions (Nebelin *et al.* 1980) or used food mixtures rather than pure compounds (Groenen *et al.* 1982; Walters *et al.* 1976). Thus it was necessary to go one step further back and predict the nitrosation rate constants of the precursors, basing the estimates on the few data that have been published (Mirvish, 1975; Ridd, 1961) and on an understanding of the reaction mechanism and the general factors that influence reactivity (such as steric hindrance and resonance forms).

Amines

The nitrosation reaction sequence (Fig. 3) is an example of a nucleophilic substitution reaction (S_N2 reaction). The electron-rich amine nitrogen attacks the nitrogen of nitrous anhydride, replacing the nitrite group, which splits off. The reactants are the unprotonated amine R_1R_2NH and two molecules of nitrous acid, to give the following kinetic equation (Turney & Wright, 1959):

$$d[NOC]/dt = k_1[R_1R_2NH][HNO_2]^2 \ldots\ldots \quad (2)$$

The most important factor determining the kinetics of amine nitrosation involves the acid–base equilibria of both amine and nitrous acid (Fig. 3a). The nitrous acid–nitrite equilibrium has a pK_a of 3.4; thus after a meal, at a stomach pH of approximately 3, about half the ingested 'nitrite' is present in the reactive, protonated form. However, at this pH, only a minute fraction of the basic amine is available as the unprotonated reactive species. The exact proportion depends on the basicity of the individual amine. The strongly basic (pK_a 9–11) simple primary and secondary amines have vanishingly small effective concentrations in the stomach (one billionth to one millionth of the concentration of the protonated species), whereas the effective concentrations of the much less basic aryl amines are appreciable (Mirvish, 1975; Ridd, 1961).

The reactivity of the various amines is also affected by steric hindrance at the amine nitrogen. The primary amines with only one alkyl group would thus be expected to react more quickly than the secondary amines. Bulky alkyl groups (for example, isopropyl or benzyl groups) also decrease the reaction rate. However, when these groups are separated from the nitrogen by a short alkyl chain (as in serotonin or phenylethylamine), they should not affect the kinetics significantly.

Finally, intramolecular catalysis by the α-carboxyl group of amino acids could increase the nitrosation rate of these compounds over that of the simple amines. The mechanism probably involves removal of an amine proton by the carboxylate ion via a five-membered ring transition state.

The kinetic equation (2) can now be rewritten to take the acid–base equilibria into account by expressing the rate in terms of the total amount of 'amine' and 'nitrite' ingested:

$$d[NOC]/dt = k_2[\text{amine}]\,[\text{nitrite}]^2. \quad \dots. \ (3)$$

Note that k_2 is pH dependent. In this form, the kinetic equation allows the various precursor types to be compared with one another with respect to nitrosatability. By comparing the structures and pK_a values of the dietary amines with amines for which k_2 values at optimal pH are known (pH range 2.5–3.4), using values taken from Mirvish (1975) and Ridd (1961), the maximal nitrosation rate constants were estimated. The k_2 values of the most important members of each class are listed in Table 4.

Amides, guanidines and ureas

The kinetics of amide-type nitrosation are different from those for the amines. The amide (and guanido and ureido) nitrogen is much less basic than an amine (pK_a 0 *v.* pK_a 10) and is normally unprotonated at stomach pH. The problem of effective concentration therefore involves only the nitrous acid–nitrite equilibrium, and thus reaction rates rise with increasing acidity of the solution. The mechanism of amide-type nitrosation (Fig. 3b) involves nucleophilic attack of the nitrogen on protonated nitrous acid. Water acts as the leaving group. The kinetic equations are as follows (Mirvish, 1971):

$$d[NOC]/dt = k_3[RNHCOR]\,[HNO_2]\,[H^+] \quad \dots. \ (4)$$

$$d[NOC]/dt = k_4[\text{amide}]\,[\text{nitrite}]\,[H^+]. \quad \dots. \ (5)$$

The reaction is first order with respect to nitrous acid, rather than second order as for the amines. This important difference means that the rate of amide-

Table 4. Approximate daily gastric *in vivo* nitrosation yields from the most abundant dietary precursors and nitrite

Compound	Daily intake (mg)	Approx. pKa	Rate constant estimate*	Yield of *N*-nitroso compound (pmol) $[NO_2]$ = 1.7 μM	$[NO_2^-]$ = 72 μM
Primary amines					
Spermidine	35	10	0.005	12×10^{-3}	24
Tyramine	21	9.1	0.05	78×10^{-3}	144
Cadaverine	15	10	0.01	14×10^{-3}	28
Putrescine	15	9.7	0.01	18×10^{-3}	32
Methylamine	3	10.7	0.005	4×10^{-3}	8
Dopamine	0.2	9.1	0.01	0.7×10^{-3}	1
Primary amino acids					
Glutamic acid	>3.2	9.7	1	>0.2	>400
Glycine	>1.3	9.6	1	>0.2	>320
Alanine	>0.4	9.7	1	$>46 \times 10^{-3}$	>80
Secondary amines					
Dimethylamine	1.7	10.7	0.002†	0.7×10^{-3}	2
Methylbenzylamine	0.6	9.5	0.013†	0.7×10^{-3}	1
Pyrrolidine	0.6	11.2	0.005†	0.4×10^{-3}	0.8
Secondary amino acids					
Proline	>0.5	9.7	0.037†	$>2 \times 10^{-3}$	3.2
Sarcosine	1‡	9.7	0.23†	27×10^{-3}	48
Arylamines					
N-Methylaniline	1.6	4.85	250†	40	68×10^3
Aniline	1	4.6	500†	57	100×10^3
Amides					
In protein	92,000	0	0.001	800	32×10^3
Carnosine	2000	0	0.001	4	160
Guanidines					
Creatine	800	—	0.004	62	2.4×10^3
Creatinine	300	—	0.004	26	1×10^3
Agmatine	0.7	—	0.004	54×10^{-3}	2
Methylguanidine	0.2	—	0.004†	0.03	1
Ureas					
Methylurea	>1	0	10.5†	>400	$>14 \times 10^3$
N-Carbamoylputrescine	1‡	0	1	20	0.8×10^3
Citrulline	1‡	0	0.7†	10	0.4×10^3

*k_2 at optimal pH for amines, k_4 at pH 2 for amide-type compounds.
†Rate constants from Mirvish (1975).
‡Arbitrary daily intake of 1 mg assigned.

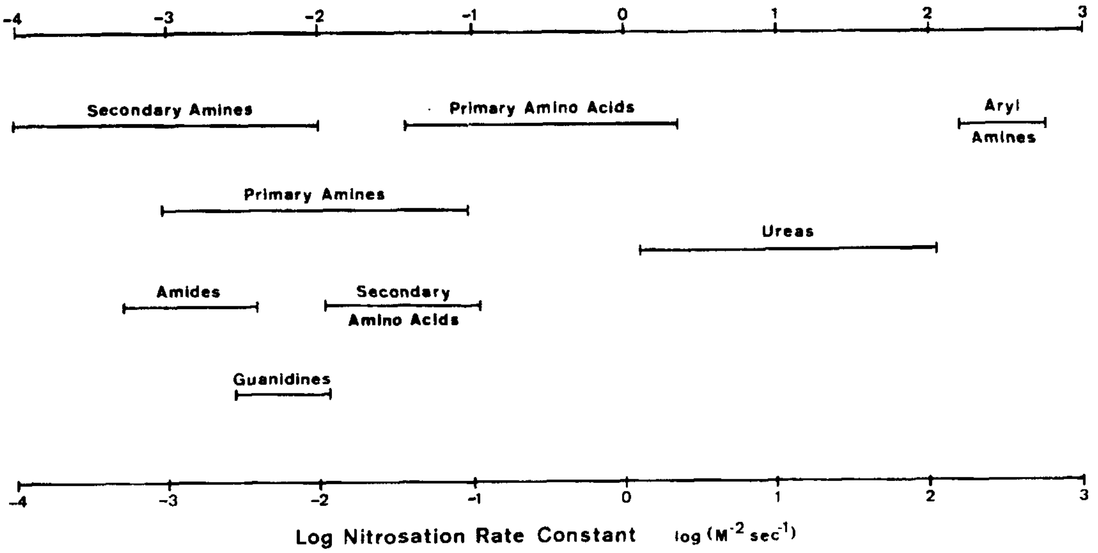

Fig. 4. Overview of nitrosation rate constant ranges of various precursor classes—k_2 (M^{-2} sec^{-1}) for amines at optimal nitrosation pH, k_4 (M^{-2} sec^{-1}) for amides at pH 2.

type nitrosation is less sensitive to the concentration of nitrite in the stomach.

The nucleophilicity of the different kinds of amide compound plays a decisive role in their reactivity. The carbonyl and protonated guanido groups are strongly electron-withdrawing. The nucleophilicity of the amide and guanidine N atoms is thus markedly reduced, and these types of compound nitrosate very slowly, with k_4 values between 10^{-3} and 10^{-2}. The presence of a second nitrogen in the urea compounds compensates for this electron-withdrawing effect, and the nucleophilicity remains strong. Reaction constants vary from 1 to 100 for these compounds. Within the reactivity range normal for each precursor type, the presence of other functional groups such as acids, amines or ring structures influence the reactivity of individual compounds. The k_4 values of the most important members of each class are listed in Table 4 (data from Mirvish, 1975).

Comment

An overview of the predicted reactivity ranges for all the precursor classes is provided in Fig. 4. The reactivities span seven orders of magnitude with arylamines and ureas highest and amines and amides lowest in the ranking order.

Nitrosation yields *in vivo*

The nitrosatability estimates from Fig. 4 were next used to calculate how much NOC would be formed in the stomach after a typical meal. No attempt was made to correct to 37°C the *in vitro* nitrosation rates determined at 25°C. The stomach volume was estimated to be 1 litre and reactants were assumed to remain at a constant concentration in the stomach for 1 hr. The total daily amount of each specific amine or amide-type compound was assumed to be eaten all at one meal. The gastric nitrite concentrations were taken from studies by Klein *et al.* (1978) and Tannenbaum *et al.* (1974). Calculations were carried out for a low nitrite burden, using [nitrite] = 1.7 μM and for a high nitrite burden, with [nitrite] = 72 μM. The pH in the stomach varies from <2 to >5, depending on the flow of gastric juice and the buffering capacity of its contents at any particular time (Walters *et al.* 1976). A pH of 2 was assumed for calculating amide-type nitrosation yields, and the optimum pH value for the nitrosation reaction was assumed in calculating nitrosamine yields (pH between 2.5 and 3.4). Given that the *in vivo* nitrosation yields span seven orders of magnitude, the errors introduced into the calculation through these assumptions are negligible.

The results of these calculations are found in Table 4. At low nitrite concentrations, the most significant yields of NOC come from protein and from methylurea (800 and 400 pmol, respectively), followed by the guanidines and arylamines (100 pmol). Because of greater sensitivity to the nitrite concentration, the *in vivo* nitrosation of arylamines becomes even more important than that of ureas plus protein at high nitrate concentrations (200 nmol versus 50 nmol). The amounts of nitrosamine produced *in vivo* from aliphatic amines are, in contrast, very small, comprising only picomole quantities even after consumption of large amounts of nitrite. The yields of *N*-nitrosamino acids lie between these two extremes, ranging from <1 pmol at low nitrite concentrations to >400 pmol at high nitrite levels.

The total gastric formation of NOCs is calculated to lie between 1 and 2 nmol/day at low and 200 nmol/day at high nitrite consumption. In comparison, the daily exposure to preformed volatile nitrosamines in food lies between 10 and 20 nmol/day (dimethylnitrosamine (DMN) 1 μg, *N*-nitrosopyrrolidine 0.1 μg; Spiegelhalder *et al.* 1980). The exposure to preformed non-volatile NOC in the diet appears to be 10–50 times higher (Kawabata *et al.* 1984), of the order of 100–1000 nmol/day. These estimates can also be compared with the total concentration of NOCs that has been measured experimentally in human gastric fluid. Two studies on fasting subjects (normal and gastritis patients) gave mean values of <100 nmol NOCs/litre (n = 50; Bartsch *et al.* 1984) and 420 nmol/litre (n = 455, range 10–40,000 nmol/litre; Walters *et al.* 1982). Fasting normal subjects had a mean of 140 nmol/litre in a further study (n = 50; Reed *et al.* 1984). The gastric juice from four non-fasting normal subjects was sampled repeatedly over the course of a day, giving a mean value of 200–4000 nmol NOCs/litre with peaks up to 18,000 nmol/litre after a meal (Bavin *et al.* 1982). The estimated total daily exogenous and endogenous NOC burden correlates well with these measurements.

The relative contribution of endogenously formed NOCs to the total daily burden thus spans a range from 'negligible' to 'the same order of magnitude as exogenous NOC' depending on the status and eating habits of the individual. On the basis of quantity, therefore, the nitrosation of food precursors in the stomach could represent a potentially important source of NOCs, particularly nitrosamides, *N*-nitrosoureas, aromatic *N*-nitrosamines and *N*-nitrosoguanidines.

Carcinogenicity

Finally, it was necessary to estimate the carcinogenic potency of the *N*-nitroso derivatives that could form in the stomach. Lifetime rodent bioassays on carcinogenicity provide the largest source of data for this purpose. Studies were selected in which the NOC was administered orally to rats. The oncogenic potency model defined by Meselson & Russell (1977) was used to convert the variables of daily dose D (mmol/kg body weight/day), treatment period t (in 2-yr units) and fraction of tumour-bearing animals I into a numerical estimate of carcinogenic potency, the OPI:

$$\mathrm{OPI} = \frac{-\ln(1-\mathrm{I})}{\mathrm{D} \times \mathrm{t}^{\mathrm{n}}} \quad \ldots. (6)$$

The variable n was assigned a value of 3, in accordance with results from Schmähl (1979) and Meselson & Russell (1977).

It must be stressed that the criteria used here to select carcinogenicity data were far less stringent than those applied by Parodi *et al.* (1982) or by Meselson & Russell (1977); consequently, the OPI values summarized in Table 5 are to be considered only as a rough guide to carcinogenic strength. The various estimates for each compound allow the substances to be divided into five categories (Table 6): 10^3, very strong; 100, strong; 10, moderate; 1, weak; <0.1, 'non-carcinogenic'.

Even with relaxed requirements there were many compounds for which no quantitative data were available. In these cases, extrapolations had to be made using the following general toxicological principles and structure–reactivity relationships:

An increase in side-chain length (R groups in Fig. 2) beyond two carbon atoms has been shown empirically to decrease carcinogenic potency (Druckrey *et al.* 1967). This may be because the larger diazonium ions are sterically hindered from reaching the active sites on the DNA, or because the resulting adducts are less stable or more easily repaired than methylated or ethylated bases. Furthermore, the addition of a charged or polar side group, such as a carboxyl, hydroxyl or amino group, also results in decreased carcinogenicity. Such structure–reactivity phenomena have been extensively studied by Lijinsky (1981b). The reason is probably that polar hydrophilic compounds diffuse only with difficulty through membranes and tend to be excreted rapidly in the urine.

Primary amines

Primary nitrosamines are intermediates in the breakdown pathway of secondary nitrosamines (see Fig. 1). They are unstable in aqueous solution and, not surprisingly, no long-term studies have been attempted on these compounds. Their behaviour in biological matrices has not been characterized. The *in vivo* nitrosation of radioactive methylamine with nitrite in rats led to measurable amounts of 7-[^{14}C]methylguanine in DNA isolated from the gastro-intestinal tract (Huber & Lutz, 1984). It thus appears that primary nitrosamines are stable enough to penetrate the cell membrane. Overall, their carcinogenic potencies may be somewhat smaller than those of the secondary amine analogues in the respective target organs. Because the effect of the chemical instability could not be quantified, OPI estimates were conservative and were based on the potency of the secondary nitrosamines. Thus, from studies on DMN and dibutylnitrosamine and on *N*-nitrosopiperidine, *N*-nitrosopyrrolidine and alkylarylnitrosamines (Druckrey *et al.* 1967), methylnitrosamine was ascribed a potency of 10^3 and the larger compounds a potency of 100.

Primary amino acids

These *N*-nitroso derivatives are also unstable and, again, no long-term studies were found. As direct-acting carcinogens in the stomach, nitrosamino acids might be predicted to have potencies similar to those of the primary nitrosamines. Nitrosoproline is here inappropriate as a reference substance because its *N*-nitroso derivative is stable and not metabolized in the body (Ohshima *et al.* 1982). *N*-Nitrosoglycine and *N*-nitrosoalanine were assigned a potency of 100, whereas *N*-nitrosoglutamic acid was assigned a potency of 10 because of its second acid group. As these are derivatives of endogenous compounds, there is also the possibility that membrane carrier systems might help the compound to gain access to cell components. The importance of this factor is difficult to assess.

Secondary amines

The secondary nitrosamines have been extensively studied. Pioneer work was carried out by Druckrey *et al.* (1967) and various members of this class were considered in the IARC Monograph series on the evaluation of carcinogenic risk to man (IARC Working Group, 1978). Hence, OPI values could be calculated for most of the compounds (Table 5). It is notable that in spite of the rather large variations in estimates from one study to the next, the OPI value

Table 5. Oncogenic potency index (OPI) estimates of *N*-nitroso compounds calculated using published data

Compound*	Daily dose (mg/kg)	Length of experiment (days)	Tumour-bearing animals (%)	Potency (OPI)	Reference
Secondary *N*-nitrosamines:					
diethyl	1–10	730	100	61	Druckrey *et al.* 1963
	0.15–0.6	730	high		Druckrey *et al.* 1963
	0.075	840	71	802	Schmähl, 1979
	14.2	68	100		Schmähl, 1979
	>0.15		100		Druckrey *et al.* 1963
dimethyl	4	730	high		Argus & Hoch-Ligeti, 1961
	4	270	65	384	Druckrey *et al.* 1967
	0.04	<840	2.7	>33	Terracini *et al.* 1967
	0.1	<840	7.4	>37	Terracini *et al.* 1967
	0.2	<840	40	>124	Terracini *et al.* 1967
	0.4	<840	65	>127	Terracini *et al.* 1967
	1	<840	83	>86	Terracini *et al.* 1967
methylbenzyl	0.25	500	50	860	Druckrey *et al.* 1967
	1	250	95		Druckrey *et al.* 1967
methylethyl	1	500	60	291	Druckrey *et al.* 1967
piperidine	5	280	90		Druckrey *et al.* 1967
	20	730	100	17	Druckrey *et al.* 1967
	3	392	82		Eisenbrand *et al.* 1980
	0.6	795	41	77	Eisenbrand *et al.* 1980
	0.12	746	8	93	Eisenbrand *et al.* 1980
pyrrolidine	5–20	290–470	92		Druckrey *et al.* 1967
	4	45–105	90		Lijinsky & Taylor, 1976
	10	444	46	27	Preussmann *et al.* 1977
	3	535	84	155	Preussmann *et al.* 1977
	1	685	32	47	Preussmann *et al.* 1977
	0.3	664	20	97	Preussmann *et al.* 1976
Secondary *N*-nitrosamino acids:					
proline	50–100	730	<5	<0.1	Druckrey *et al.* 1967
		365	0		Mirvish *et al.* 1980b
sarcosine	100	28	36	1.2	Druckrey *et al.* 1967
	200	57	50	1.1	Druckrey *et al.* 1967
Aromatic *N*-nitrosamines:					
methylaniline	10	450	80	73	Druckrey *et al.* 1967
N-Nitrosamides:					
methylbenzamide	4.4	730	88	65	Bulay *et al.* 1979
methylacetamide	1	500	100	952	Druckrey *et al.* 1967
N-Nitrosoguanidines:					
MNNG	6.6	365	73	232	Bralow *et al.* 1973
	6.6	365	10	19	Bralow *et al.* 1973
	5.9	730	45	>15	Lijinsky & Reuber, 1984
cimetidine	3	742	0	<3	Lijinsky & Reuber, 1984
N-Nitrosoureas:					
carboxymethyl†	5.2	730	60	20	Bulay *et al.* 1979
dihydrouracil	0.9	365	95	3000	Bulay *et al.* 1979
ethyl	0.4	365	62	1700	Pelfrene *et al.* 1975
	0.8–7	730	84	53	Ogiu *et al.* 1974
	2	180	95		Bulay *et al.* 1979
hydantoin	11.8	730	84	15	Bulay *et al.* 1979
hydroxyethyl	1.5	365	90	1275	Bulay *et al.* 1979
methyl	4–8	240–300	90	560	Druckrey *et al.* 1967
butyl	5	350	90	427	Takeuchi *et al.* 1984

MNNG = *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine

*Primary *N*-nitrosamines and primary *N*-nitrosamino acids are not included because no relevant data were traced in the literature.

†Hydantoic acid.

of each compound seems to be predictable to within a factor of ten. The major sources of error arose from making use of studies that lasted less than 2 years, and from the various authors' different definitions of tumour incidence. Most potencies lay between 10 and 100, with the exceptions of DMN, diethylnitrosamine and methylbenzylnitrosamine (all 10^3).

Secondary amino acids

The work on *N*-nitrosoproline (Mirvish *et al.* 1980a) and *N*-nitrososarcosine (Druckrey *et al.* 1967) showed that the presence of the carboxylic acid group greatly reduced carcinogenicity. Nitrososarcosine has a potency of about 1, and nitrosoproline appears to be virtually non-carcinogenic with a potency of <0.1.

Aromatic amines

The breakdown of aromatic nitrosamines leads to the formation of diazo compounds. Such substances are well-known reactive intermediates in organic syntheses and would be expected to react efficiently with cell components. From the one study traced, *N*-nitrosomethylaniline was calculated to have an OPI of about 100 (Druckrey *et al.* 1967). The *N*-nitroso

Table 6. Estimate of health risk posed by gastric *in vivo* nitrosation of food precursors relative to consumption of preformed dimethylnitrosamine (DMN)

Compound	Carcinogenic potency*	Health risk relative to DMN†	
		$[NO_2^-] = 1.7\ \mu M$	$[NO_2^-] = 72\ \mu M$
Primary amines			
Spermidine	100	1×10^{-7}	2×10^{-4}
Tyramine	100	7×10^{-7}	1×10^{-3}
Cadaverine	100	1×10^{-7}	3×10^{-4}
Putrescine	100	2×10^{-7}	3×10^{-4}
Methylamine	10^3	4×10^{-7}	8×10^{-4}
Spermine	100	3×10^{-8}	3×10^{-5}
Total...		10^{-6}	10^{-3}
Primary amino acids			
Glutamic acid	10	1×10^{-6}	2×10^{-3}
Glycine	100	1×10^{-5}	1×10^{-2}
Alanine	100	3×10^{-6}	8×10^{-3}
Total...		10^{-5}	10^{-2}
Secondary amines			
Dimethylamine	10^3	7×10^{-8}	2×10^{-4}
N-Methylbenzylamine	10^3	7×10^{-7}	1×10^{-3}
Pyrrolidine	100	3×10^{-9}	8×10^{-6}
Total...		$<10^{-6}$	10^{-3}
Secondary amino acids			
Proline	<0.1	$<1 \times 10^{-10}$	$<1 \times 10^{-7}$
Sarcosine	1	3×10^{-9}	5×10^{-6}
Total...		10^{-9}	$<10^{-5}$
Arylamines			
N-Methyl aniline	100	4×10^{-4}	0.8
Aniline	100	6×10^{-4}	1
Toluidine	100	3×10^{-5}	3×10^{-2}
Total...		10^{-3}	>1
Amides			
Protein	10	8×10^{-4}	4×10^{-2}
Carnosine	1	2×10^{-7}	1×10^{-5}
Total...		$<10^{-3}$	10^{-2}
Guanidines			
Creatine	1	6×10^{-6}	2×10^{-4}
Creatinine	10	2×10^{-5}	1×10^{-3}
Agmatine	10	6×10^{-8}	2×10^{-6}
Methyl guanidine	10^3	1×10^{-5}	6×10^{-4}
Total...		$<10^{-4}$	$<10^{-2}$
Ureas			
Methyl urea	10^3	$>5 \times 10^{-2}$	>1
N-Carbamoyl putrescine	100	2×10^{-5}	1×10^{-3}
Citrulline	10	1×10^{-5}	4×10^{-4}
Total...		$>5 \times 10^{-2}$	>1

*Approximate OPI of nitroso derivative.
†Risk from DMN = 1.

derivatives of the two other dietary aromatic compounds, *N*-nitrosotoluidine and *N*-nitrosoaniline, were also assigned potencies of 100, on the basis of their very similar structures.

Amides

Here again the smaller analogue, methylnitrosoacetamide (OPI about 1000) is a more powerful carcinogen than the larger methyl-*N*-nitrosobenzamide (OPI 100; Bulay *et al.* 1979; Druckrey *et al.* 1967). The potencies are analogous to those of the secondary nitrosamines. Nitrosocarnosine was assigned a potency of 1, due to its size and the presence of an acid group.

Nitrosated peptides have apparently never been tested for their carcinogenicity. One would expect the potency to be moderately low (here estimated at 10) because of their size and polarity, but to be higher if they were broken down to constituent amino acids (OPI approximately 100; see discussion of primary amino acids above). Peptides could, however, provide a relatively stable transport form of nitrosated amino acids.

Guanidines

While many studies have been conducted on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and related compounds, no data on the *N*-nitroso derivatives of dietary guanidines are available. MNNG has a potency of 10–100, whereas *N*-nitrosocimetidine has an OPI of <3 (Lijinsky & Reuber, 1984). From this meagre information, it was assumed that a nitrosated guanidine would not be more potent than the analogous nitrosated amine or amide. Methylnitrosoguanidine was thus assigned an OPI of 10^3. Nitrosoagmatine and nitrosocreatinine were assumed to be intermediate in strength between *N*-nitrosocadaverine and *N*-nitrososarcosine and given an OPI of 10. Nitrosocreatine was considered to be equivalent to *N*-nitrososarcosine (OPI 1).

Ureas

Nitrosoureas have been fairly well studied, and from Table 5 it is clear that the potencies follow the same pattern as seen with the other classes of NOCs (small alkyl > large alkyl > polar substituted). Quantitatively, however, this class appears to be more potent than the others (compare *N*-nitrosobutylurea, OPI 400, with *N*-nitrosopyrrolidine, OPI 70, for example, or *N*-nitrosohydantoic acid, OPI 20, with *N*-nitrososarcosine, OPI 1). The one compound that does not follow this pattern is *N*-nitrosodihydrouracil. Its structural similarity to thymine perhaps allows it to act either as an NOC or as a base analogue to produce DNA lesions, hence the increased oncogenic potency. The biologically important *N*-nitrosoureas, *N*-nitrosocarbamoylputrescine and *N*-nitrosocitrulline, were assigned potencies of 100 and 10, respectively.

Calculation of health risk

The health risk posed by *in vivo* nitrosation of food components was compared to that posed by the presence of preformed DMN in foods. Estimates of the health risks due to particular NOCs were calculated using equation 1:

$$\begin{aligned}\text{Risk}_{\text{NOC}} = {} & \text{daily intake of precursor C (mol/day)} \\ & \times \text{gastric concentration of nitrite}^{n} \\ & (1.7 \text{ or } 72 \times 10^{-6}\,\text{M})^{n} \\ & \times \text{nitrosatability rate constant} \\ & \text{k}_2\,(\text{sec}^{-1}\,\text{M}^{-2}) \text{ or } \text{k}_4\,(\text{sec}^{-1}\,\text{M}^{-2}) \\ & \times \text{carcinogenicity of derivative OPI} \\ & (\text{kg mmol}^{-1}\,\text{day}^{-1}\,\text{year}^{-3}) \qquad \ldots. \ (7)\end{aligned}$$

The OPI categories in Table 6 were used as estimates of the carcinogenicity. The model assumes that health risk is linearly related to both the carcinogenicity and the daily endogenous yield of each NOC. Similarly, the risk due to preformed DMN (intake 10 nmol/day; Spiegelhalder *et al.* 1980) was calculated as follows, using the best OPI estimate available (Parodi *et al.* 1982):

$$\begin{aligned}\text{Risk}_{\text{DMN}} &= \text{daily exposure to DMN} \times \text{OPI} \\ &= 10\,\text{nmol} \times 3000 = 30{,}000. \qquad \ldots. \ (8)\end{aligned}$$

The relative risk can be expressed as follows:

$$\text{Relative risk} = \text{Risk}_{\text{NOC}}/\text{Risk}_{\text{DMN}}. \qquad \ldots. \ (9)$$

Results of these calculations may be found in Table 6. Risk contributions from individual members of each compound class are listed to demonstrate the span of risk estimates within each class (up to three orders of magnitude). Tyramine, glycine, *N*-methylbenzylamine, sarcosine and aniline are the compounds with the largest individual risk contributions in each of the amine categories. Similarly, risk estimates for protein, creatinine and methylurea were calculated to be the largest within the different amide-type categories. It must be noted that this risk analysis compares isolated precursors to DMN, and whereas the total contribution of precursors is probably greater than the sums given here, the total contribution of preformed NOCs in the diet may also prove to be larger than at present imagined. A detailed picture of the types and amounts of non-volatile NOCs in the diet is lacking at present.

Figure 5 gives an overview of the relative health risks posed by *classes* of dietary *N*-nitroso precursors. The results span nine orders of magnitude with ureas and aryl amines at the top and secondary

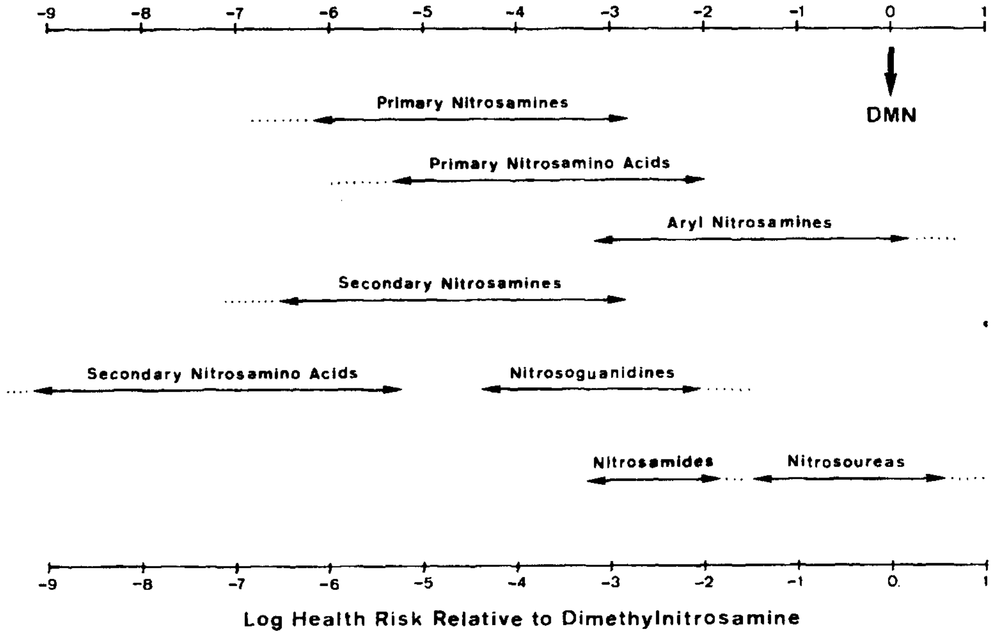

Fig. 5. Relative risk posed by dietary intake of nitrosatable precursors as compared to risk from dietary intake of dimethylnitrosamine: ▶ high nitrite concentration; ◀ low nitrite concentration; ... low pH.

amino acids at the bottom of the scale. Each line represents the risk arising from the endogenous nitrosation of a particular precursor class over a span of gastric nitrite concentrations. The risk from amine precursors is more sensitive to gastric nitrite concentration, covering three orders of magnitude, as compared to one or two orders of magnitude with the amide-type compounds. The arrows on either end of the line indicate how the risk estimate is affected by gastric nitrite concentrations below 1.7 μM (left arrow) or above 72 μM (right arrow). In addition, the effect of gastric pH below 2 is indicated by the dotted line. A low gastric pH represents an additional risk factor for amide-type compounds and arylamines, whereas it is a mitigating factor in the risk posed by alkylamines and amino acids. It is noteworthy that the risks calculated in this study are all equal to or below the risk from preformed dietary DMN.

Discussion

Attention was first given to the issue of *in vivo* nitrosation because of the extremely potent carcinogenicity of the secondary nitrosamines. It was thus natural that the environmental secondary amines were first examined as potential precursors for endogenous nitrosation (Lijinsky & Epstein, 1970; Walker, 1981). These simple amines did not cause tumours when fed concurrently with nitrite (Sander, 1971; Telling *et al.* 1976). Emphasis thus gradually shifted to the weakly basic secondary amines (synthetic compounds and drugs) and to nitrosamide-type compounds. Mirvish (1971, 1977 & 1983) has long maintained that *in vivo* nitrosation would only be a problem for rapidly nitrosatable compounds, and has thus emphasized the role of ureas as precursors. Interest has also been growing in the possible nitrosation of natural amines, peptides and proteins because of their assumed or known high concentrations in the stomach (Challis *et al.* 1984; Outram & Pollock, 1984; Piacek-Llanes *et al.* 1982; Preussmann, 1984; Walters *et al.* 1983). *In vitro* nitrosation experiments on food mixtures have also suggested that unidentified non-volatile NOC are synthesized in much larger amounts than the well-known volatile nitrosamines (Walters *et al.* 1974). Model calculations by Fine *et al.* (1982) also suggested that compound classes other than secondary amines must be more important as *in vivo* nitrosation precursors, at least as far as gastric cancer is concerned. Mutagenicity and carcinogenicity studies of nitrosamide-type compounds have revealed these substances to be activation-independent agents with a range of potencies similar to those found in nitrosamines (Bulay *et al.* 1979; Piacek-Llanes *et al.* 1982).

In our risk assessment, an attempt has been made to pinpoint areas where further research is warranted; conclusions are based not just on one criterion, such as 'high concentration in gastric juice', 'rapid nitrosation' or 'highly carcinogenic', but on the likely interactions of these several factors. The risk ranges presented in Fig. 5 show an enormous span between the highest and lowest classes of compound. Whereas the food contents of these compounds had a span of five or six, the nitrosatability rate constants seven and the carcinogenicity five orders of magnitude, the risk estimates cover nine orders of magnitude. This suggests that errors involved in the primary assumptions, even up to a factor of 10 or 100, would not seriously sway the conclusions.

This preliminary study suggests that ureas, aromatic amines and amides are the most important precursors of endogenously formed NOC. Under conditions of high gastric nitrite concentration, endogenously formed *N*-nitrosoureas and aromatic nitrosamines could pose a risk equal to or greater than that of unavoidable DMN in the diet.

Work on developing methods to identify ureas in foods (Kawabata *et al.* 1980; Mirvish *et al.* 1980b), synthesizing new *N*-nitroso derivatives of naturally occurring ureas, and testing the carcinogenicity of such compounds (Bulay *et al.* 1979) deserves to be intensified. Far too little is known about dietary ureas at present.

In contrast, the aromatic amines are relatively well known to toxicologists, because they induce methaemoglobin formation and some are established carcinogens for the human bladder. It is therefore hardly surprising that additional mechanisms of toxicity, such as the aspects discussed here, have not yet been studied in depth. On the other hand, diazo-coupling reactions are well known in the dye industry, so that one might anticipate the carcinogenic potential of nitrosated aromatic amines postulated from this study.

Nitrosated proteins and guanidines from food precursors only approach the risk levels of DMN under extreme conditions (very low stomach pH and high nitrite concentration). Normally, they constitute a risk of approximately 1% of preformed DMN. Work underway to examine the mutagenicity of *N*-nitroso derivatives of natural amides (Piacek-Llanes *et al.* 1982) should be expanded, as should kinetic studies designed to determine which side groups influence nitrosatability positively or negatively (Walters *et al.* 1974; Shephard *et al.* 1987). The stability of nitrosated peptides should be examined, along with their possible role as storage forms of nitrosated amino acids (see below).

Guanidines fall into the medium priority category. Mirvish (1971) suggested that they might be of importance because they could form *N*-nitrosoureas via an oxidative nitrosation reaction. However, in the light of the potency of MNNG and related compounds, it would seem justified to examine the contribution of *N*-nitrosoguanidines in their own right. Aside from creatine and creatinine, very little is known about the sources or reactivities of dietary guanidines.

The primary amines and amino acids have been largely ignored as nitrosatable precursors because of the instability of primary nitrosamines. This appears justified in the case of alkylamines, where the calculated risk is low in comparison to exogenous DMN; our risk estimate correlates well with a risk assessment made by Huber & Lutz (1984). Primary *N*-nitrosamino acids present a somewhat higher risk, and if nitrosated peptides prove to be a storage form of nitrosamino acids, capable of transporting *N*-nitrosamino acids into the cell, their potency as possible activation-independent carcinogens would be worth further study. The main question is the stability/reactivity of the *N*-nitroso derivatives and how this is influenced by the side chains of the various amino acids.

Weakly basic secondary amines do not appear to be found in a normal human diet; it is thus possible that secondary amines are a negligible risk factor in comparison to other precursor classes. This hypothesis correlates with *in vitro* food nitrosation studies, where resulting volatile nitrosamines (DMN, diethylnitrosamine, *N*-nitrosopiperidine, *N*-nitrosopyrrolidine) were rarely found under conditions comparable to those observed in gastric fluid (Groenen *et al.* 1982).

Finally, the secondary *N*-nitrosamino acid received a very low ranking in the risk estimates. Nitrososarcosine is weakly carcinogenic, while *N*-nitrosoproline has no carcinogenic potency (Oshshima & Bartsch, 1981). If the other secondary *N*-nitrosamino acids also prove to be excreted unchanged in the urine, their low risk ranking would appear to be justified.

Conclusions

Dietary precursors of NOC can be grouped, as follows, into classes according to the risk that arises from their endogenous nitrosation:

(i) Ureas, aromatic amines: potentially important risk factors in gastric cancer.
(ii) Amides (including protein), guanidines, primary amino acids: risk contribution uncertain.
(iii) Alkylamines (primary and secondary), secondary amino acids: most probably negligible risk factors.

Two priorities for future investigation emerge from this risk analysis. First, the sources and levels of arylamines and ureas in the diet should be studied comprehensively. This would allow a more realistic estimate of the *total* risk contributed by arylamines and ureas. Secondly, the carcinogenic potencies of key nitrosated products should be determined more precisely than the necessarily vague categories presented here. Unfortunately, the instability of some *N*-nitroso derivatives precludes their testing in long-term studies. Work is currently in progress in our laboratory to develop short-term tests; these will allow us to characterize the overall reactivity (nitrosatability of precursor and alkylating power) or genotoxicity of dietary components that form unstable NOC.

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Appendix I: Food sources of *N*-nitroso precursors

Quantities, where given, are means or ranges of the levels found—in mg of the given compound per kg of the specified food.

Primary amines

Methylamine	Spinach (12) Cabbage (3.4) Kale (16.6) Carrot (3.8) Radish (42) Maize (26.8) Apple (5.6) Cod roe (10.3) Freeze-dried coffee (16–80) Barley (4.5) Soya bean (50) Spoiled sausage Fresh pork (1.5) Pickles (1–26) Red cabbage (22.7) Cauliflower (65) White beet (17.6) Red beet (30) Celery (6.4) Lettuce (37.5) Herring (2–7) Cheese (3–12) Cocoa (60) Tea (50) Hops (3.7) Rapeseed cake (550) Green algae Bacon (0.7)
Ethylamine	Spinach (8.4) White beet (4.3) Radish (10) Maize (2.4) Apple (3) Freeze-dried coffee (1.5–2) Wine (0.5–1.7) Red cabbage (1.3) Carrots (1) Red radish (40) Lettuce (3.3) Cheese (1–4) Barley (3.4) Hops (5.2)
n-*Propylamine*	Swede (5) Pickled cucumber (7.5) Pickled onions (1.8) Cheese (2–8.7) Milk (3–10) Pickled paprika (2.3–10.6) Pickled pepperoni (1.4) Pickled celery (2.7) Brown bread (1.6)
Isopropylamine	Maize (2.3)
n-*Butylamine*	Kale (7) Cheese (3.7) Brown bread (1.1) Pickled cucumber with mustard (5.3) Soya-bean oilseed (1)
Isobutylamine	Freeze-dried coffee (1) Cocoa (6)
n-*Pentylamine*	Cabbage (1.4) Swede (1) Lettuce (3) Pickled cucumber (1.7) Herring (17) Freeze-dried coffee (10–15) Cauliflower (3.3) Red radish (6.9) Pickled paprika (1.6–3) Pickled cucumber with mustard (2.8) Cheese (1.2)
Isopentylamine	Spinach (3.8) Swede (5) Freeze-dried coffee (1) Rapeseed cake (140) White beet (7.6) Rhubarb (3.9) Cocoa (10) White wine (2–3)
Benzylamine	Spinach (6.1) Cabbage (2.8) Kale (3.8) Carrots (2.8) Red radish (4.8) Maize (3.4) Rhubarb (2.9) Red cabbage (3.3) Cauliflower (1.4) White beet (5.3) Radish (1.8) Celery (3.4) Lettuce (11.5)
Phenethylamine	Spinach (1.1) Cabbage (2.1) Kale (3) Swede (40) Rhubarb (3.2) Rapeseed cake (90) Red cabbage (8.6) Cauliflower (1.8) White beet (1.3) Carrots (2) White wine (2) Mature sausage (5)
n-*Dodecylamine*	Pickled cucumber (2.7) Pickled onions (3.6) Pickled pepperoni (6.4)
Tyramine	Cheese (27–1500) Spoiled meat Tomatoes (4) Smoked/pickled fish (470–3000) Milk (1.1) Sauerkraut (20–95) Raspberries (13–98)

Appendix I: Food sources of *N*-nitroso precursors

Quantities, where given, are means or ranges of the levels found—in mg of the given compound per kg of the specified food.

	Ham (2–8) Spoiled fish Bananas (7) Beer (2–11) Meat (0–1200) Octopus Mature sausage (60–564) Wine (3) Pork (11) Avocados (23)
Histamine	Cheese (1) Fish (1–30) Smoked pork (4–5) Spinach (38) Sausage (60–140) Milk (0.4) Spoiled fish Pork (6) Sauerkraut (6–100) Tomato (22) Wine (7) Bacon (1–15)
Cadaverine	Cheese Peas (6–7) Smoked pork (14–630) Sausage (15–174) Spoiled fish Soy sauce (200) Pork (170) Sauerkraut (3–30)
Putrescine	Cheese Soy sauce (80) Mature sausage (90) Pork (170) Spoiled fish Smoked pork (11–500) Bacon (2–36) Sauerkraut (1–40)
Spermidine	Pork (420) Soya beans Endogenous in mammalian tissue Smoked pork (150–1270) Bacon (3) Ham (4)
Dopamine	Bananas (8)
Ethanolamine	Ham (4–6) Bacon (10–22) Pork (8) Sauerkraut (2–7)
Tryptamine	Tomatoes (4) Pork (14) Bacon (3–13) Cheese (0–1100) Sausage (10) Ham (8–67)
Serotonin	Bananas (28) Pineapple (17–65) Tomatoes (12)
Hexylamine	Milk (5–17)
Spermine	Pork (61) Smoked pork (6–800) Bacon (1–37) Ham (51)

Amino acids

Glutamic acid	Milk Meat (large amount) Cheese Grain Eggs Mushrooms (1300)
Glycine	Milk Meat (large amount) Mushrooms (200)
Aspartic acid	Milk Meat (moderate amount) Mushrooms (200)
Alanine	Milk Pork (large amount) Mushrooms (1300)
Serine	Milk Meat (large amount) Mushrooms (300)
Glutamine	Meat Mushrooms (400)
Arginine	Grain Mushrooms (900)
Leucine	Grain Mushrooms (400)
Proline	Milk (1.5) Mushrooms (1000)
Sarcosine	Lobster Cartilaginous fish

Secondary amines

Dimethylamine	Red cabbage (2.8) Cauliflower (14) Red radish (1.1) Maize (26.8) Pickled cucumber (15.4) Herring (3.4–7.8) Freeze-dried coffee (3–6) Soya-beans (8) Pork (0.1–0.2) Octopus with soya (369) Cabbage (2) Kale (5.5) Celery (5.1) Lettuce (7.2) Pickled onions (1) Brown bread (3.1) Barley (1.6) Hops (1.4) Sardines in oil (180) Salmon (48) Mackerel (26)
Diethylamine	Spinach (15) Pickled cucumber (1.4) Herring (1.9–5.2) Barley (5.7) Beef (2×10^{-4}) Apple (3) Pickled onion (3.2)

Appendix I: Food sources of *N*-nitroso precursors

Quantities, where given, are means or ranges of the levels found—in mg of the given compound per kg of the specified food.

	Cod roe (5.2) Hops (3.1) Spoiled fish
Methylethylamine	White beet (7.6) Maize Freeze-dried coffee (1–2) Carrots (7) Lettuce Herring (1) Hops (3.7)
Pyrrolidine	Spinach (2.5) Maize (3.5) Pickled cucumber (5.6) Pickled onions (8.4) Cheese (1–20) Hops (1) Malt (1.5) Red radish (38) Pickled paprika (1.4) Pickled pepperoni (1.8) Pickled celery (2.6) Freeze-dried coffee (7–10)
Pyrroline	Red radish (20)
Piperidine	Pickled paprika (5.2) Pickled celery (1) Cocoa (9) Barley (1) Beef (1×10^{-3}) Pickled pepperoni (3.4) Freeze-dried coffee (1–2) Hops (2.5) Pepper
N-*Methylbenzylamine*	Lettuce (10) Carrots (16) Herring (2)
N-*Methylphenethylamine*	Cauliflower (1.6) White beet (1.6) Carrots (2) Red radish (5.4) Rhubarb (2.6) Pickled cucumber (2.2) Pickled onions (6.5) Cheese (2.6) Spinach (2.4) Kale (2) Swede (2) Radish (6.6) Maize (1.1) Apple (1.2) Pickled cucumber with mustard (7.3) Red cabbage (3.7)
(*Morpholine*	Solvent in food industry)
Chavicine	Pepper

Arylamines

Aniline	Red cabbage (1) Cauliflower (22) Carrots (30.9) Red radish (4.6) Apple (1.5) Cabbage (4) White beet (1.2) Radish (2.8) Rhubarb (5) Rapeseed cake (120)
Toluidines	Kale (1.1) Celery (1.1) Carrots (7.2)
N-*Methylaniline*	Spinach (3.4) Pickled cucumber (13.8) Pickled celery (7) Pickled paprika (13.1) Pickled onions (6.8) Cheese (37.9)

Amides

Asparagine	Carrot juice Asparagus
Glutamine	Meat
Pyrrolidone carboxylic acid	From glutamate
Glutathione	Liver Muscle
Carnosine	Muscle (90–4600) Fish
Anserine	Muscle Poultry

Guanidines

Guanidineacetic acid	Mammalian blood
Creatine	Vertebrate muscle (5000) Fish muscle (7000)
Methylguanidine	Dried fish products (20–180) Sardines (60–1900) Cod Milk Liver Shark Beef
Agmatine	Shellfish (<650) Cheese Abalone
Creatinine	Dried fish (4100) Bacon (3300) Fried beef (540)

Ureas

Citrulline	Watermelon Green peppers Soy sauce Mushrooms
Albizziin	Mimosa
(β-(p-*Nitrophenyl*)-*ureidopropionic acid*	Sweetener)
N-*Carbamoylputrescine*	Grains
Allantoic acid	Plants
Hydantoic acid	Sugar-beet sprouts
N-*Carbamoylaspartic acid*	Intermediate in pyrimidine biosynthesis

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Original Contribution

Generation of NO by probiotic bacteria in the gastrointestinal tract

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Abstract

Probiotic bacteria elicit a number of beneficial effects in the gut but the mechanisms for these health promoting effects are not entirely understood. Recent in vitro data suggest that lactobacilli can utilise nitrate and nitrite to generate nitric oxide, a gas with immunomodulating and antibacterial properties. Here we further characterised intestinal NO generation by bacteria. In rats, dietary supplementation with lactobacilli and nitrate resulted in a 3–8 fold NO increase in the small intestine and caecum, but not in colon. Caecal NO levels correlated to nitrite concentration in luminal contents. In neonates, colonic NO levels correlated to the nitrite content of breast milk and faeces. Lactobacilli and bifidobacteria isolated from the stools of two neonates, generated NO from nitrite in vitro, whereas *S. aureus* and *E. coli* rapidly consumed NO. We here show that commensal bacteria can be a significant source of NO in the gut in addition to the mucosal NO production. Intestinal NO generation can be stimulated by dietary supplementation with substrate and lactobacilli. The generation of NO by some probiotic bacteria can be counteracted by rapid NO consumption by other strains. Future studies will clarify the biological role of the bacteria-derived intestinal NO in health and disease.
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Keywords: Nitrite; Nitrate; Allergy; Colitis; Lactobacilli; Newborn infants

Introduction

It has been suggested that dietary supplementation with live microorganisms, known as probiotics (e.g. lactobacilli and bifidobacteria), may be beneficial for the host. Acting either themselves or by influencing the host flora, probiotics may enhance various defence mechanisms, modulate innate and adaptive immunity, eliminate toxins, carcinogens and pathogens, release antioxidants and stimulate gastrointestinal motility [1]. The molecular mechanisms governing these activities are currently the subject of intense investigation. It seems clear that both agents secreted by metabolically active viable probiotics (bactericions, short-chain fatty acids and peptides) and components from nonviable bacteria (DNA, protein constituents) can mediate protective pathways, but for many of these effects the exact mechanism of action still remains to be elucidated.

Nitric oxide (NO) is a free radical gas involved in numerous physiological and pathophysiological events in the gastrointestinal tract. Endogenous NO generated from L-arginine by NO synthases (NOS) serves to regulate mucosal blood flow, mucus generation, water and electrolyte transport, motility and host defence responses. In addition, NO synthesis is upregulated during inflammatory conditions [2,3]. We and others have been studying an alternative NOS-independent route for NO generation in the gut that involves commensal bacteria [4–7]. Oral commensal bacteria reduce salivary nitrate to nitrite which then spontaneously decomposes to NO and other nitrogen oxides in the acidic stomach [8,9]. In a recent study, we found that isolated strains of lactic acid producing bacteria generated NO in vitro when nitrite was added to the growth medium [10]. Again, NO generation was a result of pH reduction, this time caused by the bacteria themselves via formation of lactic acid. All together, this has led to the speculation that NO generated locally in the intestinal lumen can mediate some of the beneficial effects of probiotic bacteria [7,10,11].

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At birth, intestinal NO concentrations are very low (<10 parts per billion, ppb) in humans [12]. Once the amniotic membranes are ruptured, bacterial colonization of the neonatal intestinal tract begins. In association with exposure to increasing numbers of gut bacteria, intestinal NO in healthy newborn infants increase rapidly to, in some cases, very high (>1000 ppb) levels [12]. The origins and physiological role of this neonatal peak in intestinal NO are unclear.

In the process of revealing the biological significance of NO formed in the GI tract it will be important to understand how the generation is controlled. The aim of the present study was to investigate if commensal bacteria can generate NO in vivo in the gut and to further explore the importance of substrate (nitrate/nitrite) availability. We first studied if dietary supplementation with a live lactobacilli strain and inorganic nitrate would generate NO in rats. In addition, we measured colonic NO during the first days of life in newborn infants; here the aim was to study the relation between the NO levels in the colon and the amounts of nitrate and nitrite in ingested breast milk. Finally, we also investigated in vitro generation and consumption of NO by different strains isolated from newborns.

Material and methods

Animal experiments

Adult male Wistar rats (n=36, weight 325±15 g) were divided into four groups: control group, (Control), rats fed with *Lactobacillus rhamnosus* (LGG, ATCC 53103; Valio Ltd., Helsinki, Finland) for 7 days, rats fed with nitrate (NO_3^-), and rats fed LGG together with nitrate (LGG+ NO_3^-). Either sodium nitrate ($NaNO_3$, 0.1 mmol/kg/day) or the same amount of NaCl (Control) was given in distilled drinking water. LGG was anaerobically cultered in MRS medium (de Man, Rogosa and Sharp, Merck, Darmstadt, Germany) at 37°C for 24 hours. Then 1 ml (10^9 CFU/ml) of the culture was given orally to each animal and the remaining contents (10 ml) were spread on the fur and bedding material. The animals were kept at standard conditions (t=21±2°C, 12 h light/12 h darkness). They received a rodent diet, (TD 99366 chow for rats, Harlan, USA) found to be very low in nitrate.

At the day of the NO measurements, 120 mg/kg of sodium pentobarbital anaesthetic was administered intra-peritoneally, followed by laparotomy. Luminal NO gas measurements were performed as described [13,14]. Briefly, using a 5 ml syringe with a thin needle, NO-free air (<3 ppb) was directly inflated into the caecum, stomach, small intestine and colon. The NO-free air was obtained by sampling room air (NO<10 ppb) via charcoal filter. After incubating the air for 15 sec in the different compartments the intestinal gas was aspirated and immediately injected into a chemiluminescence analyser (Aerocrine AB, Stockholm, Sweden) and the peak NO concentration was measured. The instrument's detection limit for NO was 1 ppb. Calibration of the instrument was performed with cylinder gas (10 ppm NO in nitrogen; AGA AB, Lidingö, Sweden).

Samples of intestinal contents from the small intestine, caecum and colon were tested for nitrate and nitrite concentrations: 0.5 mg wet weight was diluted in 0.5 ml sterile 0.9% NaCl and centrifuged to separate faecal material from a clear supernatant. The supernatant was stored at −20°C and later analysed with chemiluminescence after reductive cleavage and subsequent determination of the NO released into the gas phase as described in detail recently [15]. The detection limit for nitrate/nitrite in this assay is <10 nM. At the end of the experiments the rats where killed with an overdose of sodium pentobarbital given intra-peritoneally.

Studies in newborn infants

A total number of 34 healthy, newborn infants (14 girls/20 boys), delivered either vaginally (n=24) or by elective Caesarean section (n=10), were included in this study (gestational age 38±2 w, birth weight 3718±510 g, mean±SD values). Exclusion criteria were maternal antibiotic therapy during the last month of pregnancy, preterm birth, asphyxia at birth, signs or symptoms of neonatal disease, malformations or lack of informed parental consent.

The study protocol included a single measurement of intestinal NO at 3-6 days after birth. Colonic gas samples were collected using a tonometric balloon technique described recently [12]. Briefly, an all-silicone catheter (Sherwood Medical, Tullamore, Ireland) equipped with an inflatable balloon tip was inserted 8-10 cm into the sigmoid colon via rectum. The balloon was inflated with 5 ml NO-free air and left to equilibrate in the intestine for 5 min. After the incubation period, the gas was aspirated and immediately injected into the chemiluminescence NO analyser determined and expressed in parts per billion (ppb).

During the investigation faeces was collected after spontaneous defecation and treated with aseptic precautions. To analyse nitrite and nitrate, 0.5 g wet weight was diluted in 0.5 ml sterile 0.9% NaCl and centrifuged (20, 000 rpm for 2 h) to separate faecal material from a clear supernatant. Supernatant was stored at −20°C for later analysis of nitrate and nitrite.

All infants were breastfed and 5 ml of the breast milk was collected and frozen at −20°C for later analysis of nitrate and nitrite concentrations. The sample of breast milk was taken at the day of NO measurements.

In vitro experiments with bacteria

Using fresh faeces from two of the healthy neonates, simple colonies with typical appearance were isolated and identified by conventional bacteriologic methods as *Lactobacilli* sp, *E. coli*, *Bifidobacterium* sp and *Staphylococcus aureus*. For lactobacilli and bifidobacteria no further species identification was performed. Bacteria were inoculated anaerobically at 37°C for 24–48 hours, on different agars supplemented with 0,1 mM $NaNO_2$ as described recently [10]. Briefly, 100 μl (about 10^9 CFU/ml) of the precultured inoculates was put on either lactobacilli agar AOAC (pH=6.8, Difco, USA) for lactobacilli and bifidobacteria. Alternatively, we used ISO-sensitest agar plates, (pH=7.0, Oxoid, Basingstoke, England), to culture *E. coli* and *S. aureus*. After inoculation, the plates were inserted into 500 ml gas tight infusion bags, from Cryovac (Infubags®, S.E. Nündel Kunststoff-Technik GmbH, Germany) together with an anaerobic pouch system and an anaerobic indicator (AnaeroGen™ compact AN0020C, and BR0055, Oxoid, Basingstoke, England). Thereafter the bags were sealed and 300 ml air was injected. After 1 hour, when anaerobic conditions were achieved in the bag, the anaerobic pouch inside the bag was sealed off with an impulse sealer. In some experiments lactobacilli and bifidobacteria were monoinoculated on the plates and in other a combination of two bacterial strains were used; lactobacilli or bifidobacteria together with either *S. aureus* or *E. coli.* In the later cases, the two different strains were inoculated separately (on one half each) on AOAC plates. The control experiments were performed with the same agar plate but without bacteria present. After 1, 6, 9, 18 and 24 hours of incubation at 37°C, 10 ml gas was aspirated from the gas-tight bag. The NO concentration of this gas sample was immediately measured with the rapid-response chemiluminescence system.

To directly study NO consumption, *S. aureus* was inoculated on the agar plates and after 16 hrs of anaerobic growth at 37°C, NO gas (final concentration 1000-1400 ppb NO) was injected into the infusion bags. Subsequently, 10 ml gas sample was aspirated after 30 min and again at eight hours and NO levels were analysed as described above.

Ethics

Two study protocols (animal and human studies) were reviewed and approved by the Regional Ethics Committee for animal and human experimentation, respectively. The performed studies complied with all applicable federal guidelines and institutional policies.

Statistics

Statistical analysis and graph plotting were performed with Prism 4.0 (GraphPad Software, San Diego, California, USA). NO values are given as median (range) and group differences were tested with Mann-Whitney test. Correlation was analyzed with the Spearman rank test. A *p*-value of less than 0.05 was considered significant.

Results

Intestinal NO generation can be stimulated by dietary supplementation with substrate (nitrate) and lactobacilli in vivo

The combination of nitrate and LGG increased NO both in the small intestine ($p<0.001$) and the caecum ($p<0.001$), exceeding those of control animals by 3-8 times. LGG alone increased NO levels in the caecum ($p<0.05$) whereas dietary nitrate alone did not affect intestinal NO. Gastric NO increased dramatically by nitrate alone as described earlier while LGG did not affect these levels (Table 1, Fig. 1).

Measuring nitrate and nitrite in the contents of the different GI compartments showed that the rats supplemented with nitrate, alone or in combination with LGG, had significantly higher nitrite-levels in the small intestine ($p<0.05$) and in the caecum ($p<0.05$, Table 1, Fig. 2). In addition, nitrite levels measured in the caecum correlated to the ceacal NO levels, ($r=0.73, p<0.01$).

Colonic NO levels in newborn infants in relation to nitrite content of breast milk in neonates

When levels of NO were measured in the colon of neonates using the tonometric balloon technique, the median of intracolonic NO levels 3-6 days after birth was 60 (2–1760) ppb. This is similar to the levels, described in a previous study [12] also showing that NO levels peaked at day 3–5. The nitrite concentration in breast milk at the time of the measurement was 0.1 (0–1.1) μM and the corresponding nitrate concentration was 51 (24–169) μM. Concentrations of nitrite and nitrate in faeces were 0.6 (0.1–7.7) and 28.0 (13–130) μM, respectively. Intracolonic NO levels correlated to nitrite in breast milk ($r=0.46, p<0.01$) (Fig. 5) and in faeces ($r=0.45, p=0.02$), but not to nitrate levels.

NO generated by lactobacilli and bifidobacteria can be consumed by E. coli or S. aureus

From the faecal contents of two of the healthy neonates, simple colonies with typical appearance were isolated and identified by conventional bacteriologic methods as lactobacilli

Table 1
Effects of dietary supplementation with live lactobacilli and/or nitrate on the levels of nitric oxide gas (NO), nitrite (NO_2^-) and nitrate (NO_3^-) measured in the gastrointestinal tract of rats

	Stomach			Small intestine			Caecum			Colon		
	NO (ppb)	NO_2^- (μM)	NO_3^- (μM)	NO (ppb)	NO_2^- (μM)	NO_3^- (μM)	NO (ppb)	NO_2^- (μM)	NO_3^- (μM)	NO (ppb)	NO_2^- (μM)	NO_3^- (μM)
Control	190 (43–500)	nd	nd	36 (6–62)	2 (1–3)	121 (59–153)	40 (17–500)	0 (0–1)	58 (34-81)	12 (4–15)	1 (0–1)	84 (53–158)
LGG	107 (35–210)	nd	nd	38 (12–160)	4 (1–6)	102 (56–115)	83 (38–280)	1 (0–2)	57 (43-94)	8 (6–22)	2 (1–4)	167 (80–357)
NO_3^-	10,550 (2900–26,000)	nd	nd	77 (11–280)	16 (5–64)	82 (38–173)	52 (27–310)	4 (0–8)	30 (17-104)	14 (5–42)	1 (0–11)	82 (53–120)
LGG+ NO_3^-	6080 (190–9980)	nd	nd	130 (22–260)	13 (5–18)	177 (64–181)	316 (185–1410)	4 (2–7)	79 (47-85)	12 (3–30)	3 (0–16)	89 (76–101)

Median (range) * 0=<0,1 μM, nd=not determined.

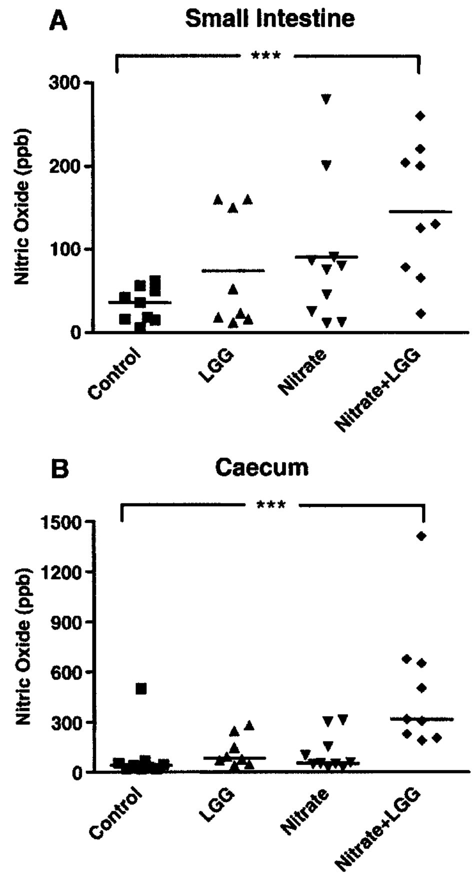

Fig. 1. Nitric oxide levels in the small intestine (A) and caecum (B) of rats fed with *Lactobacillus rhamnosus* (LGG), $NaNO_3$ 0,1 mmol/kg/day (Nitrate), *Lactobacillus rhamnosus* + nitrate (LGG + Nitrate) and controls (Control). NO was measured directly in luminal gas.

sp., *E. coli*, bifidobacterium sp. and *S. aureus*. When grown on agar plates supplemented with nitrite in sealed plastic bags, the isolated lactobacilli and bifidobacteria, but not *E. coli* nor *S. aureus* generated NO, up to 1200 ppb after 16 hours. These findings corroborate our previous studies [10] showing that commercial strains of lactobacilli (e.g LGG) and bifidobacterium can generate NO in vitro. In contrast, when the isolated lactobacilli or bifidobacteria were co-incubated with *E. coli* or *S. aureus* the total level of NO did not exceed 300 ppb, suggesting that the NO generated by the isolated strains was consumed by the *E. coli* and *S. aureus* (Fig. 3). In addition, when exogenous NO gas was added to the gas tight bag, *S. aureus* consumed added NO, reducing it from 1300 ppb to 2 ppb in less than 8 hrs (Fig. 4).

Discussion

We show that NO generation locally in the gut can be enhanced by dietary supplementation with live lactobacilli and nitrate. In this pathway nitrate is most likely reduced step-wise by bacteria to nitrite and then to NO. We also demonstrate that NO generated by some gut bacteria can be effectively consumed by other microbes. Thus, the levels of NO present in the gut reflect the combined balance between the formation and consumption of NO.

The mechanism of NO formation from nitrate and nitrite differs somewhat in the GI compartments but the presence of bacteria is always required. This is obvious from studies in germ-free rats where NO is low throughout the GI tract [14]. Gastric NO generation has been well characterised and the very high NO levels found there is a result of two prevailing conditions; a high acidity and abundance of substrate [4,9]. As shown here and earlier [13,16], gastric NO increases greatly after an oral nitrate load. This is because more nitrate is extracted from blood by the salivary glands, secreted with saliva and eventually reduced to nitrite by the oral microflora before entering the acidic stomach [4]. While nitrate loading alone increased gastric NO, the addition of live lactobacilli did not seem to influence the levels. This is expected, as lactobacilli are not critical for oral nitrate reduction [17].

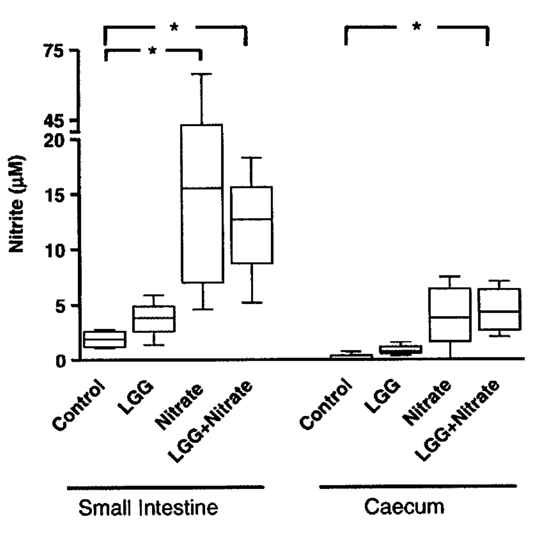

Fig. 2. Nitrite levels in the small intestine and caecum of rats fed with *Lactobacillus rhamnosus* (LGG), $NaNO_3$ 0,1 mmol/kg/day (Nitrate), *Lactobacillus rhamnosus* + nitrate (LGG + Nitrate) and controls (Control). Nitrite was measured in luminal contents.

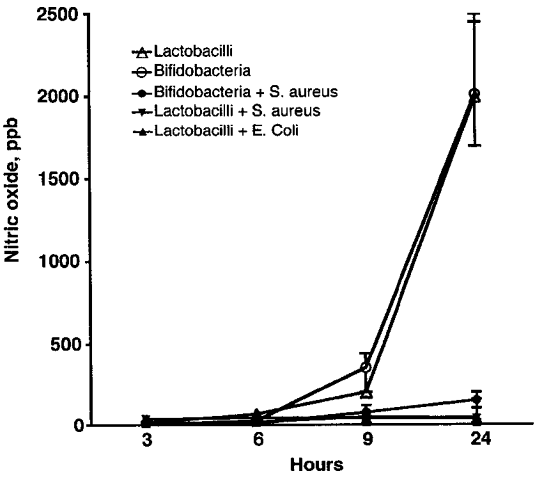

Fig. 3. Generation of nitric oxide by lactobacilli and bifidobacteria inoculated alone or together with *S. aureus* and *E. coli*. Bacteria were grown anaerobically on plates placed in gas-tight bags. At indicated time points, a gas sample was drawn from the bag and NO was measured. All experiments were done in triplicate.

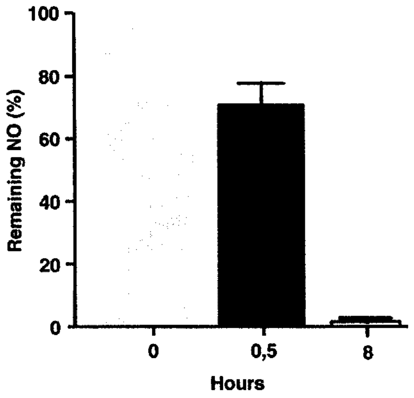

Fig. 4. Consumption of NO by *S. aureus* as compared with a control agar plate without bacteria. Bacteria were grown anaerobically in gas-tight bags and NO gas (1300 ppb) was injected into the bag at the start of the experiment. NO was measured in gas samples taken at indicated time points.

As the intragastric NO generation has been fairly well studied [4] the major aim of the present study was to characterise NO generation by bacteria also in the lower parts of the GI tract. We found that NO levels in the small intestine and the caecum were 3-8 fold higher in animals that had been fed live lactobacilli and nitrate compared to controls. In rats given lactobacilli or nitrate alone, NO also increased slightly but to a lesser extent. How then is NO formed in the small intestine and in the caecum? Nitrate supplementation increases salivary nitrite as discussed above and parts of this nitrite may reach the small intestine and caecum. Indeed, the levels of nitrite were much higher in intestinal contents of nitrate fed rats (Fig. 2) compared to controls and the amounts correlated to the NO levels. Alternatively, nitrate could be reduced to nitrite locally in the lower parts of the intestines by commensals residing in that area. Further reduction of nitrite to NO could proceed via at least two possible mechanisms. First, many bacteria have nitrite reductase enzymes which generate NO from nitrite [4,18]. Denitrification (the step-wise anaerobic reduction of nitrate to nitrogen gas with intermediate formation of NO) is widespread among bacteria in nature although for example enterobacteria do not utilise this pathway. Second, NO can be formed non-enzymatically from nitrite via acid-dependent mechanisms as in the stomach. In vitro studies seem to support the latter explanation. Thus, lactobacilli generate NO from nitrite in vitro as shown here and earlier [7,10] and this NO formation is closely correlated to a pH reduction in the growth medium. In addition, in lactobacilli enzymatic nitrite reduction is a rare property and many strains are completely devoid of nitrite reductase [34]. Alternatively, one might argue that lactobacilli increase NO by stimulating the mucosa to generate NO from NO synthase. However, NO levels did not increase by lactobacilli or nitrate alone (it required the combination of the two) so this seems unlikely. In addition, in germ-free rats no increase in NO was noted after dietary supplementation with nitrate [14] indicating that challenge with this anion per se does not lead to induction of NOS activity in the host mucosa.

In the colon levels of NO were low and did not increase after supplementation with lactobacilli or nitrate. It is likely that nitrite and nitrate is absorbed or consumed to a large extent before reaching the colon and therefore the lack of available substrate may explain this finding. Indeed, in this study neither nitrate nor nitrite increased in the colon after dietary supplementation with nitrate. Alternatively, the lactobacilli may not have established in that particular GI compartment. A third interesting possibility is that any generation of NO in the colon is effectively counteracted by a rapid consumption of NO. Indeed, we show here that other commensals (*S. aureus* and *E. coli*) can consume NO generated by lactic acid producing bacteria. When these bacteria were co-incubated in vitro with NO generating species (lactobacilli or bifidobacteria) the normal increase in NO was completely prevented (Fig. 3). Moreover, when exogenous NO gas was added to cultures of *E. coli* or *S. aureus* it was rapidly consumed (Fig. 4). Bacteria have developed several ways of removing potentially toxic NO from their vicinity. These include nitrite reductases and NO reductases, which reduce NO to less toxic nitrogen compounds [4,18], or may involve proteins (e.g. flavohemoglobins) that bind and destroy NO [4]. The in vitro model used here could be useful to further explore how NO is consumed by different gut commensal bacteria.

In the present study and in an earlier study [12] we also measured colonic NO in newborn babies in vivo using a minimally invasive tonometric method developed in our laboratory [19,20]. We now wanted to correlate NO levels with nitrate and nitrite content in breast milk and in faeces. Such dietary correlations are much easier to perform in newborns than in adults when the infants are fed exclusively breast milk. NO levels did indeed correlate with nitrite in breast milk and faeces, again indicating that nitrite can be a substrate for intestinal NO generation by bacteria (Fig. 5).

In the earlier study we found that colonic NO was uniformly low immediately after birth and then increased gradually during the first days of life [12]. In many infants we noticed a transient peak in NO on day 3-5 after birth. We speculated that this was a natural host-response involving induction of NO synthase in the mucosa in response to the emerging bacteria. A possible explanation for the transient nature of the peak could be that emerging bacteria effectively consume NO thereby creating a

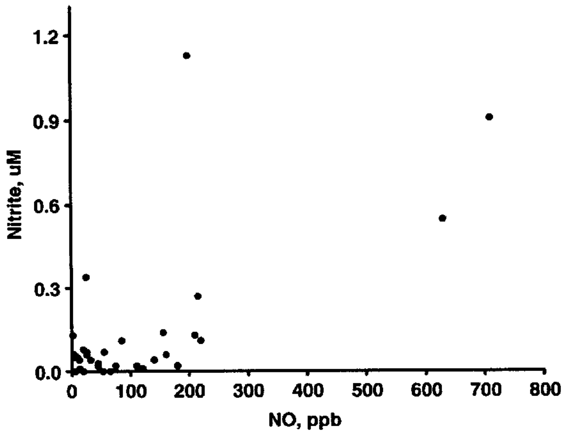

Fig. 5. Correlation between nitrite levels in breast milk and colonic NO in newborn infants ($n=34$) measured on one occasion during days 3–6 after birth.

steady-state. Indeed, almost all neonates have high counts of *E. coli* and Staphylococci in their stools already at day 3 after delivery [21].

Because newborn infants lack an established microflora, they cannot utilise nitrate directly for generation of NO so they would need preformed nitrite. The nitrate from the breast milk would probably be excreted from the body unchanged as mammalian cells cannot effectively metabolize this anion [4]. Interestingly, even the nitrite they receive directly from breast milk is derived from bacteria. In this case it is nitrate-reducing commensals inhabiting the lactating ducts of the mothers breast [22]. During the first 3-5 days after birth, there is physiologic increase of nitrate concentration in breast milk [23] and this coincides with initial NO peak in the gut.

In the present study we also measured and found both nitrate and nitrite at considerable levels in faeces of newborn infants. These levels were high as compared with faeces of adults (micro- vs nanomolar range, C Reinders et al. unpublished data). This could be due to the fast passage in the neonatal tract, which would allow nitrite to reach the colon. Nitrite delivery to the stomach will result in an immediate great NO release due to the acidity. The amount of NO formed further down will then depend on how much nitrite that "survives" gastric passage. In newborn infants intragastric pH is much higher and therefore more nitrite will probably leave the stomach unaltered. This may help to explain the correlation between nitrite in breast milk and colonic NO levels in the infants.

A fundamental remaining issue relates to the role of the NO generated in the gut by probiotics and other commensals. Is NO just an inert spill-over product from bacterial nitrogen metabolism or can it serve to regulate physiological processes? Judging from the known biological properities of NO, it is not unreasonable that some of the positive effects attributed to probiotics can be explained by formation of NO by these bacteria as has been suggested [7,10,11]. Examples of typical probiotic effects, which could be accounted for by NO or related species include immunomodulation and control of pathogen growth [1]. For the upper part of the GI tract there is clear evidence that nitrite-derived NO affects vital biological processes [13] and nitrite-derived NO stimulates gastric mucosal blood flow and mucus generation in vivo, and in vitro studies show that acidified nitrite can effectively kill gut pathogens [8,24]. In the lower parts of the GI tract the importance of this system is less clear to date. However, it is not unlikely that effects will be revealed in this area as well. NO is an extremely potent biological mediator that operates effectively already in the nanomolar range. One should also consider the possibility that other bioactive compounds are generated from nitrite locally in the gut. A classical example is nitrite-dependent formation of nitrosamines by bacteria [25], which has been implicated in carcinogenesis. Other examples include nitrosation reactions (yielding for example highly bioactive S-nitrosothiols) [13] and possibly nitration reactions to yield potentially bioactive nitrated lipids [26]. Finally, effects of the absorbed gut-derived nitrite outside the intestine can also be foreseen [4–6,15,27–33]. As an example nitrite has potent cytoprotective effects in the heart [28–31], and can also act as a vasodilator [32,33], likely via systemic reduction to NO. Thus, the intriguing possibility exists that commensal bacteria contribute to systemic formation of bioactive NO via metabolism of dietary nitrate [4,15,28,29].

We conclude that intestinal NO generation can be stimulated in vivo by dietary supplementation with substrate (nitrate) and live lactobacilli. The generation of NO by some bacteria can be counteracted by rapid consumption by other strains. Future studies will reveal if intestinal generation of NO and related species can explain any of the health promoting effects of lactobacilli and other probiotics.

Acknowledgments

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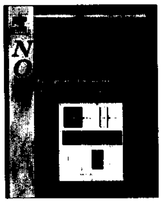

Dietary nitrate in Japanese traditional foods lowers diastolic blood pressure in healthy volunteers

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ABSTRACT

Background: Japanese longevity is the highest in the world. This is partly explained by low occurrence of cardiovascular diseases, which in turn is attributed to the Japanese traditional diet (JTD). Recent research demonstrates that nitric oxide (NO), a key regulator of vascular integrity, can be generated from nitrate (NO_3^-), abundantly found in vegetables. It can reduce blood pressure (BP) via its serial reduction to nitrite (NO_2^-) and to bioactive NO. Interestingly, JTD is extremely rich in nitrate and the daily consumption is higher than in any other known diet.

Objective and design: In a randomized, cross-over trial we examined the effect of a 10-day period of JTD on blood pressure in 25 healthy volunteers. Traditional Japanese vegetables were encouraged to be consumed and avoided during the control period. Daily nitrate intake was calculated.

Results: Nitrate naturally provided by the JTD was 18.8 mg/kg/bw/day, exceeding the Acceptable Daily Intake by five times (ADI, 3.7 mg/kg/bw).

Plasma and salivary levels of nitrate and nitrite were higher at the end of the JTD period. Diastolic BP decreased on average 4.5 mm Hg during JTD compared to the control diet ($P = 0.0066$) while systolic BP was not affected. This effect was evident in normotensive subjects and similar to that seen in the recent studies.

Conclusions: An ordinary nitrate rich diet may positively affect blood pressure. Our findings further support the importance of the role of dietary nitrate on BP regulation suggesting one possible explanation of healthy aspects of traditional Japanese food.

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Background

At an age when the average European person is predicted to die – 77 years for men and 81 for women – inhabitants of Okinawa in Japan usually have many more years of good health. Not only do the Japanese live longer, they age successfully, are lean, energetic and have low occurrence of chronic illnesses like heart disease and cancer [1]. This has partly been attributed to Japanese traditional diet rich in vegetables and fish. A typical Japanese meal consists of a rice dish complimented with soybean products, fish, seafood, and a variety of vegetables. Among the vegetables eaten every day, there are a variety of green leafy vegetables, mushrooms and seaweed. Interestingly, the population who lives longest (Okinawans), has the highest consumption of kombu (seaweed) in Japan [2]. Furthermore, the vegetable diet pattern in Japanese is associated with a significantly lower blood pressure, and serum triacylglycerides [3]. Specific foods that could reduce cardiovascular diseases have recently been identified [4–6] but more research is obviously required to identify what particular components in fruit and vegetables are associated with this decrease.

NO is a key regulator in vascular integrity. Recently a fundamentally different pathway for NO generation in addition to the classical NO synthase-dependent pathway has been described. NO can be generated from inorganic nitrate and nitrite, abundantly found in green leafy vegetables [7,8]. In humans, after absorption in the upper gastrointestinal tract, approximately 25% of circulating nitrate is actively taken up by the salivary glands and is concentrated up to 20-fold in saliva. Once in the oral cavity, commensal bacteria on the dorsal surface of the tongue reduce nitrate to nitrite by the action of nitrate reductase enzymes [8–10]. Swallowed nitrite is then reduced to NO and other bioactive nitrogen oxides in the acidic environment of the stomach. Nitrite that survives the acid conversion can enter the systemic circulation and increase its storage pool in blood and tissues. Studies in humans show increased plasma nitrite concentrations after oral ingestion of nitrate and use of an antibacterial mouthwash after

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consumption of dietary nitrate attenuates the rise in plasma nitrite, showing the importance of the oral bacteria in the nitrate conversion to nitrite [11].

However, beyond this "prokaryotic pathway" of nitrite generation in blood and tissue an "eukaryotic pathway" has also been recently described by Lundberg's group: also mammalian cells are capable of nitrate reduction to nitrite via the involvement of a nitrate reducing enzyme, XOR [12]. Nitrite accumulation in blood and tissues represent a biological pool for NO generation since several different mammalian enzymes and metalloproteins possess nitrite reductase activity such as xanthine oxidoreductase (XOR), aldehyde oxidase (AO), heme proteins and mitochondrial respiratory chain enzymes [11–13].

The measurable biological effects of nitrate derived NO include rapid local vasodilatation and acute reduction in blood pressure [5,13]. It also enhances gastroprotection [14–16], plays a role in mitochondrial respiration [17], cardiac function [18] and exerts antiapoptotic effects [19].

Significant physiologic benefits may be associated with the dietary nitrate. The content of inorganic nitrate in certain vegetables and fruits can provide a physiological substrate for reduction to nitrite and NO that produces vasodilatation, decreases blood pressure and supports cardiovascular function [20–22].

The Dietary Approaches to Stop Hypertension (DASH) studies found that diets rich in vegetables can lower blood pressure to levels similar to those achieved with single hypotensive medications [23,24]. This protection has been attributed to the high content of antioxidants, yet large clinical trials have failed to provide evidence in support of this theory [25,26]. The strongest protection against coronary heart disease was associated with the consumption of green leafy vegetables (e.g., spinach, lettuce) [27]. These vegetables commonly have a high inorganic nitrate content [28]. Interestingly, the BP reduction described after ingestion of beetroot juice decreased BP only if saliva was continuously swallowed, demonstrating the critical involvement of an enterosalivary circulation of nitrate for its bioactivation [5]. Other foods rich in nitrate–nitrite are mushrooms and seaweed. Asian population, especially Japanese, consume a diverse range of mushrooms and seaweed on a daily basis. Overall, the traditional Japanese diet contains a great number of green, leafy vegetables, making it exceptionally rich in nitrate, and the daily consumption higher than in any other known diet. We therefore aimed to examine if the Japanese traditional food, reflected in ingestion of dietary nitrate, affects plasma nitrate/nitrite and arterial blood pressure.

Experimental procedures

The 25 participants of the study were physically active, healthy Japanese volunteers (10 men and 15 women; mean age 36 ± 10 years, BMI < 18.5). They gave informed consent and the study was granted full ethics approval by the Local Research Ethics Committee at Kyorin University School of Medicine and was registered at clinicaltrials.gov, NCT 00928824. The study had a randomized cross-over design with two dietary intervention periods during which the subjects received either Japanese or control (non-Japanese) diet. The exclusion criteria were any serious illnesses, infectious diseases and use of systemic medication. Study subjects had an overnight fast on the morning of saliva and blood collection and blood pressure recording. There were two smoking participants and they were instructed not to change their smoking habits during the study period.

Common Japanese vegetables, identified as the daily source of nitrate [29,30], were encouraged to consume during the consequent 10 days. To avoid the concentration differences in nitrate/nitrite in locally produced foods, the participants were provided with fresh vegetables and staple foods from the same store twice a week during both intervention periods. During the control period, participants were instructed to avoid these vegetables. Instead, the study subjects received and followed the instructions how to replace traditional Japanese meals; for example, by having cornflakes, muesli, yogurt or sandwiches for breakfast. The control diet was designed to eliminate the risk of any major differences between diets in total protein, carbohydrates, saturated and unsaturated fat. The control diet was controlled for nitrate and nitrite sources and those were excluded from the consumption. The mean total intake of energy was approximately 1900 kcal per day. Each participant was asked to recall and fill in all food and drink items consumed daily during the study periods of 10 days, including detailed information about recipe ingredients. Daily nitrate/nitrite intake calculations were based on the dietary recall, body weight information and referred nitrate/nitrite concentrations of ingested foods [29]. Nitrate contents in various vegetables and Japanese foods (examples are shown in Table 1) were based on the calculations by Tsuji et al. (assessed by ion exchange HPLC–UV chromatography) [29]. Blood pressure was measured manually in a sitting position by the blinded physician according to a standard protocol. There was no wash-out period between the study periods. Measurements were taken before breakfast three times: at baseline, at the shift of diets in the middle and at the end of the study. Blood samples (3 mL) were collected and treated for plasma nitrate and nitrite measurement according to procedure previously described in detail [31]. Blood samples were centrifuged immediately at 2200g for 10 min at 4 °C and the plasma stored at −80 °C until measurement of nitrite and nitrate concentration. Saliva samples were collected at the same time in tubes containing EDTA (final concentration 2 mM) and stored at −80 °C for later nitrate and nitrite determination. Samples were analyzed for nitrite and nitrate concentrations by ion chromatography (ENO 20 Analyzer; Eicom, Kyoto, Japan). Sample concentrations reflect the mean value from triplicate analyses.

The data were analyzed using the Graph Pad Prism Software. Group differences were tested with Mann–Whitney (independent groups) and Wilcoxon's signed rank (paired measurements) tests. In all cases, $P < 0.05$ was considered statistically significant.

Results

Healthy Japanese individuals participating in the study followed proposed dietary schemes without significantly loosing or gaining body weight (mean [±SD], 58.7 ± 9.3 at the start of the trial, 58.9 ± 9.1 kg after the non-Japanese and 58.7 ± 9.3 after the Japanese diet). The trial subjects did not express any inconvenience fol-

Table 1
Example of nitrate levels (NO_3^-, mg/kg) in some typical Japanese foods included in daily diets, based on dietary recall [29].

Vegetable	$NO3^-$, mg/kg
Ta cai	5670 ± 1270
Chin gin cai	3150 ± 1760
Garland chrisantemum	4410 ± 1455
Osaka shirona	2500 ± 753
Spinach	3560 ± 552
Burdock	2350 ± 438
Sayaingen beans	945 ± 141
Chinese cabbage	1040 ± 289
Winter mushrooms	983 ± 93
Honghimeji mushrooms	1836 ± 48
Shiitake mushrooms	454 ± 38
Purple laver	2825 ± 2200
Laver	3990 ± 3940
Nozavana pickles	2170 ± 35
Water dropwort	504 ± 187

lowing the Japanese diet; on the contrary, it was associated with an old-fashioned Japanese diet consumed at participants' parents/grandparents homes. At the same time it was difficult to follow the control diet. Therefore, a dietary expertise was used to adjust the control diet and to ensure its nitrate levels to be within the ADI range. Individually consumed daily nitrate intake was approximated to a mean concentration of 18.8 mg/kg of body weight/day during the Japanese diet study phase. Nitrate, naturally derived from Japanese diet exceeded five times the Acceptable Daily Intake (ADI = 3.7 mg/kg/body weight).

After 10 days of Japanese diet, the circulating plasma nitrate levels were higher than after period of control diet (mean [±SD], 43.2 ± 17.4 and 153.9 ± 149 μM, respectively; $P < 0.001$), as were plasma nitrite levels (131.5 ± 75.34 and 203.5 ± 102.3 nM, respectively; $P = 0.0063$) (Fig. 1). Fasting salivary nitrate levels were (median (range), 569.6 (14.4–5778) μM after Japanese diet ($P = 0.0008$) and 199.7(0.1–703.7) μM after control; nitrite levels were 134.2 (1.2–1411) μM at the end of the Japanese diet, and 71.9 (0.4–453.2) μM at the end of the control phase $P < 0.0018$.

The mean diastolic blood pressure was 4.5 mm Hg lower after Japanese diet compared with non-Japanese diet, 71.3 ± 7.9 and 75.8 ± 7.8, $P = 0.0066$) (Fig. 2). There were no significant differences in systolic blood pressure (data not shown).

Discussion

Until recently, it has been commonly agreed that NO in vivo could only be synthesised by NOS with nitrite and nitrate as inert biological end-products of NO metabolism. However, it was demonstrated in 1994, that nitrite derived from dietary nitrate was a substrate for NOS-independent generation of NO in the acidic condition of the human stomach [8,9]. Despite the demonstration of a pharmacological role for nitrite in vascular and immune function, the potential health aspects of food sources of nitrates and nitrites have not received much attention [22].

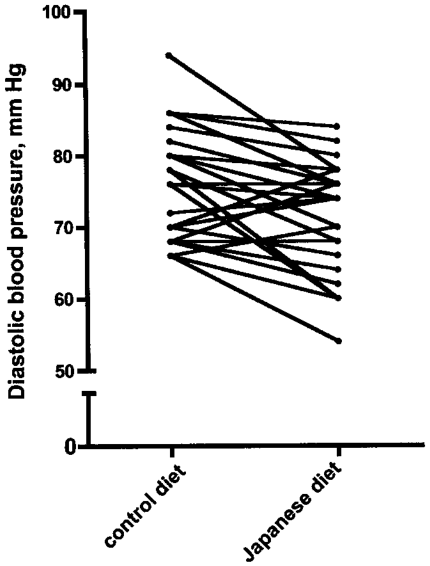

Fig. 2. Effects of 10-Day Japanese traditional foods or control diet on diastolic blood in 25 healthy volunteers. The mean diastolic pressure was by 4.5 mm Hg lower during Japanese traditional food intake ($P = 0.0066$).

The WHO reported in 2002 that the harmful effects of chronic hypertension stand for the ca 11% of all following diseases. Identifying dietary components that might protect against cardiovascular diseases will therefore be important for public health worldwide. Nitrate has been highlighted to be such a component [5,14]: administration of sodium nitrate (0.1 mmol/kg/d) to healthy volunteers over 3 days reduced diastolic BP by 3.7 mm Hg [13] and Webb an co-workers showed similar effects with a vegetable juice rich in nitrate [5]. In the present study, ordinary Japanese diet increased intravascular stores of nitrite probably

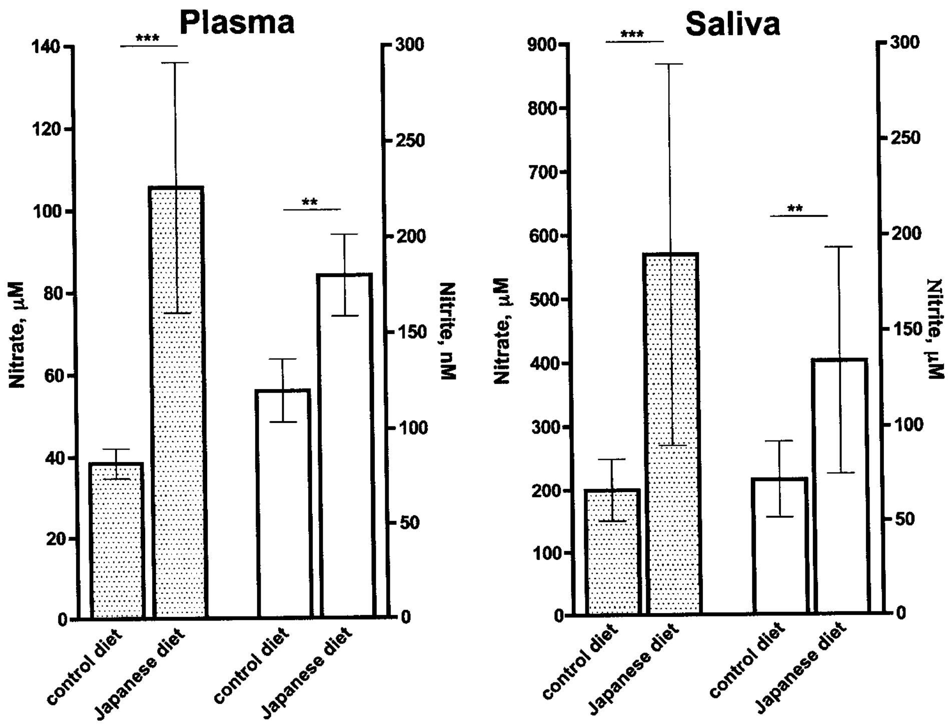

Fig. 1. Effects of 10-Day Japanese traditional foods or control food on salivary and plasma nitrate and nitrite in 25 healthy volunteers. After overnight-fasting (10 h), plasma nitrate and nitrite concentrations were higher during Japanese traditional diet ($P < 0.001$ and $P < 0.05$).

due to bioconversion. As a result, the BP decreased, because the nitrite was further converted to a potent vasodilator, NO [32]. Blood pressure decrease in normotensive Japanese volunteers was similar to that seen in the Webb and Larsen studies [5,13,23] and suggests that NO provided in the form of dietary nitrate, found in the Japanese traditional diet, would likely have a cardioprotective effect.

It is argued that the BP lowering effect of Japanese foods could be attributed to antioxidants, vitamins, polyphenols and high K^+ content of fruit and vegetables [33], although recent large scale clinical trials have failed to provide evidence in support of this hypothesis [25,26]. At the same time, nitrite reduction to NO is greatly enhanced by reducing compounds such as vitamin C and polyphenols, both of which are abundant in the Japanese foods and in the DASH diet.

Further, Webb an co-workers elegantly showed that the lowering effect of vegetable juice on BP was independent of K^+ levels, since the rise in plasma K^+ was unaffected by spitting, while nitrate effect on BP were abolished by these procedure [5]. Moreover, since dietary sodium nitrate supplementation in the present study has similar effects as shown by Larsen et al. [13], this convincingly suggests that it is nitrate and not antioxidant, polyphenols or potassium that is responsible for the BP effect.

In the present study the amount of nitrate naturally provided by the Japanese diet exceeded the ADI by four times and could therefore be questioned. Although seemingly high, these levels were easy to reach when the participants ate vegetables that corresponded to a typical traditional Japanese diet. Green leafy vegetables present in Japanese food (chingensai, komatsuna and garland chrisantemum etc.) contain on average a similar amount of nitrate as European spinach, and Japanese are high consumers of a variety of mushrooms and seaweed, also rich in nitrate/nitrite (Table 1). The variety and amounts of nitrate rich vegetables eaten every day in the traditional Japanese diet is much greater than in a European diet: almost all the foods shown in Table 1 were included in the daily diet, which corresponded an ordinary Japanese diet. Altogether, these eating habits explain the high daily intake of nitrate. Nitrate intake from dietary sources in our study is similar with the recent report from Bryan's group, who has calculated that the DASH diet could result in the consumption of up to 1222 mg nitrate per day thereby exceeding by 550% the WHO's ADI for nitrate in adults [34]. The concentration of nitrate in a single vegetable species varies depending on the soil and growth as well as storage and transport conditions [35]. In our study, we handled the possible variation in concentrations by providing the participants with the foods from the same store.

Some nitrogenous compounds, such as nitrate, since long have been and still are considered potential human health hazards; especially when given to infants, nitrates in bacterially contaminated well water could be reduced to nitrite and cause the condition known as methemoglobinemia [20]. However, the exposure studies on children and adults have not confirmed that nitrate intake is associated with methemoglobinemia [36,37], and alternative explanations for methemoglobinemia in infants has been suggested such as gastroenteritis and associated iNOS-mediated production of nitric oxide induced by bacteria contaminated water [38]. The data supporting the toxicity of nitrates and nitrites for healthy adolescent and adult populations is questionable, as is the scientific basis for exposure regulations for nitrate and nitrite [20,21,39]. Another issue, especially in Japan, is that ingested nitrites may react with secondary amines or *N*-alkylamides to generate carcinogenic *N*-nitroso compounds (NOCs) [40] and the prevalence of gastric cancer in the Japanese population is very high. Although shown in animal models [41], the proof in humans has not been substantiated. Furthermore, the nitrites in foods may be "neutralized" if accompanied by vitamin C, an antioxidant that inhibits the nitrosation effect of nitrites on secondary amines [42]. Clearly, more research is needed to address whether nitrate and nitrite intake is associated with increased cancer risk.

Today we are facing a paradigm shift, and the recent description of the vasoprotective, blood pressure-lowering, and antiplatelet aggregation properties of nitrite suggests that a re-examination of these health effects would be beneficial [5,43,44]. Of the recent studies describes increased plasma nitrite and nitrate concentrations of natives in the high-altitude of Tibet as a natural physiology not associated with harmful physiological effects [45]. The DASH diet study led to the public dietary health recommendations in the United States [46].

We are aware of our study's limitations and the findings therefore should be generalized cautiously. First, the sample size was small. Second, the compliance was difficult to access, since the dietary recalls do not always guarantee accuracy due the risk of underreporting. Despite of this, the strength of the present study is that it is an ordinary diet and could therefore be recommended as a preventive strategy if further confirmed in longer studies also including subjects with cardiometabolic risk factors.

We conclude that Japanese traditional diet, rich in nitrate reduces diastolic blood pressure in healthy volunteers. Our results show effects of an easy to follow, diverse diet and suggest a possible explanation of healthy aspects of Japanese food. By highlighting the daily nitrate and nitrite contents of vegetables our study strengthens the existing evidence to advise vegetable consumption for health benefits. Time might have come to re-evaluate the ADI recommendations regarding nitrate consumption.

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Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate

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Stead, Lori M., Keegan P. Au, René L. Jacobs, Margaret E. Brosnan, and John T. Brosnan. Methylation demand and homocysteine metabolism: effects of dietary provision of creatine and guanidinoacetate. *Am J Physiol Endocrinol Metab* 281: E1095–E1100, 2001.—*S*-adenosylmethionine, formed by the adenylation of methionine via *S*-adenosylmethionine synthase, is the methyl donor in virtually all known biological methylations. These methylation reactions produce a methylated substrate and *S*-adenosylhomocysteine, which is subsequently metabolized to homocysteine. The methylation of guanidinoacetate to form creatine consumes more methyl groups than all other methylation reactions combined. Therefore, we examined the effects of increased or decreased methyl demand by these physiological substrates on plasma homocysteine by feeding rats guanidinoacetate- or creatine-supplemented diets for 2 wk. Plasma homocysteine was significantly increased (~50%) in rats maintained on guanidinoacetate-supplemented diets, whereas rats maintained on creatine-supplemented diets exhibited a significantly lower (~25%) plasma homocysteine level. Plasma creatine and muscle total creatine were significantly increased in rats fed the creatine-supplemented or guanidinoacetate-supplemented diets. The activity of kidney L-arginine:glycine amidinotransferase, the enzyme catalyzing the synthesis of guanidinoacetate, was significantly decreased in both supplementation groups. To examine the role of the liver in mediating these changes in plasma homocysteine, isolated rat hepatocytes were incubated with methionine in the presence and absence of guanidinoacetate and creatine, and homocysteine export was measured. Homocysteine export was significantly increased in the presence of guanidinoacetate. Creatine, however, was without effect. These results suggest that homocysteine metabolism is sensitive to methylation demand imposed by physiological substrates.

methionine; methyl balance; methyltransferases; hepatocytes; liver; kidney

A NUMBER OF EPIDEMIOLOGICAL STUDIES have confirmed a relationship between an increased plasma concentration of homocysteine and the development of vascular disease (for review, see Ref. 27). A meta-analysis performed by Boushey et al. (6) of 27 studies showed that homocysteine was an independent, graded risk factor for atherosclerotic disease. Total plasma homocysteine values of ~10 μM for men and ~8 μM for women are considered normal. However, as little as a 5-μM increase in total plasma homocysteine is associated with an increased risk of coronary artery disease of 60% for men and 80% for women. The mechanism by which homocysteine exerts pathological effects is currently unknown.

During the course of its metabolism, methionine is adenylated by the enzyme *S*-adenosylmethionine synthase to form *S*-adenosylmethionine (SAM), the methyl donor in virtually all known biological methylation reactions (25). For example, the methylation of DNA and RNA and the conversion of glycine to sarcosine all require SAM as methyl donor. The end products of these methyltransferase reactions are a methylated substrate and *S*-adenosylhomocysteine (SAH), which is reversibly hydrolyzed to homocysteine and adenosine. Homocysteine has several possible fates: *1*) catabolism to cysteine via the pyridoxal phosphate-dependent transsulfuration enzymes cystathionine β-synthase (CβS) and cystathionine γ-lyase (CγL) (24), *2*) remethylation to methionine via cobalamin-dependent methionine synthase or betaine:homocysteine methyltransferase (12), and *3*) export to the extracellular space.

It is apparent, therefore, that biological methylations and homocysteine metabolism are intimately linked and that alterations in one may affect the other. Such an effect has been documented in studies of patients with Parkinson's Disease undergoing treatment with L-3,4-dihydroxyphenylalanine (L-Dopa). This disorder is characterized by an extreme depletion of nigro-striatal dopaminergic neurons that results in deficiencies of dopamine in the basal ganglia and of melanin in the substantia nigra. Treatment involves administration of L-Dopa alone or in combination with a peripheral decarboxylase inhibitor (9). The decarboxylation of L-Dopa in the brain alleviates the dopamine deficiency. In the course of treatment, a wasteful peripheral methylation of L-Dopa by catechol *O*-methyltransferase occurs with the production of 3-*O*-methyldopa, which is excreted. The result of this metabolic removal of L-Dopa is a therapy that requires very high

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doses of the drug. Consequently, methyl status is altered. Decreases in whole blood levels of SAM have been observed in L-Dopa-treated patients (9) as well as increases in plasma homocysteine in humans and rats (1, 23). It is clear that alterations in methylation demand by pharmacological agents can affect plasma homocysteine levels.

In light of this, an examination of the effects of creatine and guanidinoacetate (GAA) on homocysteine metabolism is warranted, because the methylation of GAA to creatine via GAA methyltransferase consumes more SAM than all other methylation reactions combined (34). Figure 1 describes the interorgan metabolism of GAA and creatine and their interaction with homocysteine metabolism. We undertook a series of experiments, in vivo and in vitro, to determine whether manipulation of methylation demand by the more physiological substrates, creatine and GAA, could affect homocysteine metabolism. The results described herein demonstrate that homocysteine metabolism is sensitive to the methylation demands imposed by physiological substrates.

MATERIALS AND METHODS

Animals, diets, and chemicals. Male Sprague-Dawley rats weighing between 250 and 300 g were used throughout the study. The animals were obtained from our institute's breeding colony and were housed and treated in accordance with guidelines of the Canadian Council on Animal Care (7). Memorial University's Institutional Animal Care Committee approved all procedures. All animals had free access to water and food. Rats were fed either chow or a 20% casein-based AIN-93 diet, designed to meet the nutritional requirements for growth of laboratory animals, for 2 wk before experimentation. Where indicated, diets were supplemented with 0.4% wt/wt creatine monohydrate or 0.36% wt/wt GAA. These levels were chosen because it has been previously shown that they downregulate the renal L-arginine:glycine amidinotransferase (21). Where diets were supplemented, an equivalent mass of cornstarch was omitted from the diet. Rats were housed at 22°C and exposed to a 12:12-h light-dark cycle, with the light cycle commencing at 0800. All experiments were performed immediately after the end of the dark cycle. All chemicals were purchased from Sigma Chemical (Oakville, ON, Canada), except where noted in the text.

Tissue preparation and analysis. Animals were anesthetized with pentobarbital sodium (65 mg/kg ip). After a midline abdominal incision, blood samples were collected from the abdominal aorta into heparinized syringes and placed on ice until plasma was separated by centrifugation (15 min, 3,700 *g*). Plasma was frozen at −20°C for later analysis. Kidneys and liver were rapidly removed and homogenized in ice-cold 50 mM potassium phosphate buffer (pH 6.9) with a Polytron (Brinkmann Instruments, Toronto, ON, Canada) for 20 s at 50% output. The homogenates were centrifuged at 18,000 *g* for 30 min at 4°C, and the supernatant was retained. All enzyme assays were carried out on this 18,000-*g* supernatant. All enzyme assays were demonstrated to be

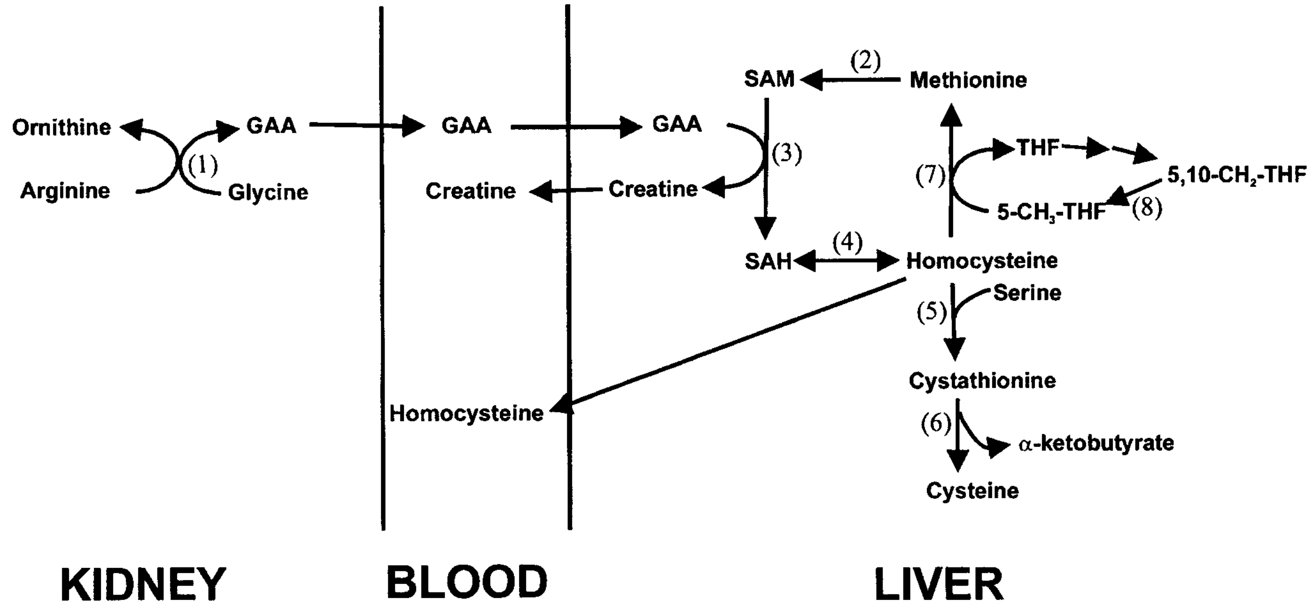

Fig. 1. Creatine synthesis: interactions with hepatic homocysteine metabolism. The first step in creatine synthesis occurs in the kidney with the transfer of the amidino group of arginine to glycine to yield ornithine and guanidinoacetate (GAA) via L-arginine:glycine amidinotransferase (1). GAA enters the circulation for transport to the liver, where it is a substrate for *S*-adenosyl-L-methionine:guanidinoacetate *N*-methyltransferase (3). *S*-adenosylmethionine (SAM), formed from methionine via *S*-adenosylmethionine synthase (2), donates its methyl group to the amidino group of GAA to yield creatine and *S*-adenosylhomocysteine (SAH). Most creatine is exported to the circulation for transport to muscle. *S*-adenosylhomocysteine, however, is reversibly hydrolyzed to adenosine (not shown) and homocysteine via *S*-adenosylhomocysteine hydrolase (4). Homocysteine has several possible fates: *1*) catabolism to cysteine via the pyridoxal phosphate-dependent transsulfuration enzymes cystathionine β-synthase (5) and cystathionine γ-lyase (6), *2*) remethylation to methionine via cobalamin-dependent methionine synthase (7) by use of 5-methyltetrahydrofolate supplied by the 5,10-methylenetetrahydrofolate reductase reaction (8) as methyl donor or via the betaine-homocysteine *S*-methyltransferase (not shown), which uses betaine as methyl donor, and *3*) export to the extracellular space.

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linear with time and with protein under the conditions employed.

For the assay of muscle metabolites, hindlimb skeletal muscle was freeze-clamped and stored at −70°C for later analysis. Frozen tissue, still in liquid N_2, was ground with a pestle and mortar, and the powder was homogenized in 4 volumes of 6% (wt/vol) $HClO_4$. Extracts were centrifuged at 35,000 *g* for 15 min at 0°C. Supernatants were decanted and neutralized with 30% KOH. Muscle and plasma creatine (4), muscle phosphocreatine (20), and adenine nucleotides (16) were assayed as previously described.

Analytical procedures. The following enzymes, responsible for the remethylation of homocysteine, were measured in liver: methionine synthase (19), methylenetetrahydrofolate reductase (11), and betaine-homocysteine *S*-methyltransferase (32). The enzymes involved in the catabolism of methionine to cysteine were also measured: CβS (22) and Cγ-L (29). For betaine-homocysteine *S*-methyltransferase and CβS assays, methionine and cystathionine, respectively, were measured by HPLC (18). The L-arginine:glycine amidinotransferase was assayed in kidney using the method of Van Pilsum et al. (30), in which ornithine is detected by ninhydrin. Protein concentration was determined using the Biuret method (13) after solubilization with deoxycholate and with bovine serum albumin as a standard (17).

Total plasma homocysteine[1] concentrations were determined by reverse-phase HPLC and fluorescence detection of ammonium 7-fluoro-2-oxa-1,3-diazole-4-sulphonate (SBDF) thiol adducts by use of the method of Vester and Rasmussen (31). For plasma methionine analysis, the samples were deproteinized with 10% sulfosalicylic acid, the protein was removed by centrifugation, and the supernatant was adjusted to pH 2.2. The samples were then analyzed on a Beckman 121 MB amino acid analyzer with a Benson D-X, 0.25 Cation Xchange Resin and a single column, three-buffer lithium method as per Beckman 121MB-TB-O17 application notes. Results were quantitated using a Hewlett-Packard computing integrator model 3395 A after postcolumn derivatization with ninhydrin.

Preparation and incubation of isolated hepatocytes. Hepatocytes were isolated as previously described (5), and viability was assessed by 0.2% trypan blue exclusion. Viability was ≥95% in all cases. Hepatocytes were preincubated for 20 min at 6–8 mg dry weight of cells/ml in a final volume of 1 ml in Krebs-Henseleit medium containing 1.25% bovine serum albumin. At the end of the preincubation, substrates were added, and the incubation was allowed to continue for an additional 30 min. Cells were gassed with 95% O_2-5% CO_2 at the beginning of the incubation and at the addition of substrates.

When homocysteine export was measured, the cells plus incubation medium were immediately centrifuged at 14,000 *g* (Brinkman Instruments, Rexdale, ON, Canada) for 2 min to sediment the cells, and an aliquot of the supernatant was removed for exported thiol analysis. This involves reduction of all thiols with 10% tri-*N*-butylphosphine in dimethylformamide, followed by deproteinization with perchloric acid and derivatization with SBDF (see *Analytical procedures*).

Statistics. Data were analyzed by ANOVA followed by the Newman-Keuls multiple comparison posttest. In all cases, $P < 0.05$ was taken to indicate significant difference.

Table 1. *Effect of GAA and creatine supplementation on hindlimb skeletal muscle metabolites and plasma creatine*

	Control	GAA	Creatine
Creatine	9.6 ± 0.9^{a}	13.3 ± 2.2^{b}	14.0 ± 2.4^{b}
Phosphocreatine	12.2 ± 2.2^{a}	13.1 ± 1.1^{a}	15.2 ± 2.3^{a}
Total muscle creatine	21.8 ± 1.8^{a}	26.4 ± 1.8^{b}	29.2 ± 1.3^{c}
Plasma creatine	0.06 ± 0.02^{a}	0.35 ± 0.04^{b}	0.31 ± 0.04^{b}
ATP	5.5 ± 0.1^{a}	5.4 ± 0.3^{a}	6.2 ± 0.5^{b}
ADP	0.96 ± 0.10^{a}	0.90 ± 0.05^{a}	0.96 ± 0.07^{a}
AMP	0.066 ± 0.005^{a}	0.091 ± 0.046^{a}	0.091 ± 0.031^{a}

Rats were maintained on control, guanidinoacetate (GAA)-, and creatine-supplemented 20% casein AIN-93 diets for 2 wk as described in MATERIALS AND METHODS. Muscle creatine, phosphocreatine, total creatine, and adenine nucleotides are given in values of μmol/g wet muscle weight. Plasma creatine is given as mM. Values are means ± SD; $n = 5$ for control and GAA-supplemented groups; $n = 4$ for the creatine-supplemented group. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with $P < 0.05$ taken to indicate significant difference. Data with differing superscripts are significantly different within rows.

RESULTS AND DISCUSSION

Effect of creatine and GAA supplementation. Table 1 describes the effect of creatine and GAA supplementation on muscle metabolites in hindlimb skeletal muscle and on plasma creatine. Muscle creatine was increased by 39% in GAA-supplemented animals and by 46% in the creatine-supplemented group compared with control values. Phosphocreatine was unchanged. Plasma creatine was about sixfold higher in both the GAA- and creatine-supplemented groups. These data indicate that dietary supplementation with creatine or GAA significantly alters both muscle and plasma creatine levels.

Table 1 also shows the adenine nucleotide content of the hindlimb skeletal muscle. No statistically significant differences in ADP or AMP were noted among groups. However, ATP was slightly elevated in the creatine-supplemented group.

Total plasma homocysteine was shown to be sensitive to dietary manipulation of methyl acceptors (Fig. 2). Animals maintained on a GAA-supplemented diet exhibited a plasma homocysteine level that was 49% higher than control animals. Supplementation of diets with creatine, however, resulted in a 27% decrease in plasma homocysteine. Plasma methionine was unchanged.

Effect of GAA and creatine supplementation on selected liver and kidney enzymes. Several enzymes of interest to homocysteine and GAA metabolism were assayed (Table 2). The two enzymes of the transsulfuration pathway, which together catalyze the catabolic removal of homocysteine from the methionine cycle, were assayed in liver. The activity of the first enzyme, CβS, was 89% higher in the GAA-supplemented group than in the creatine-supplemented group. However, neither group differed significantly from the control group. The activity of liver CγL, the final enzyme of the transsulfuration pathway, was unchanged among groups.

Three enzymes required for the remethylation sequence of homocysteine, methionine synthase, methyl-

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[1] "Total plasma homocysteine" refers to the sum of free and protein-bound homocysteine, as well as mixed disulfide forms.

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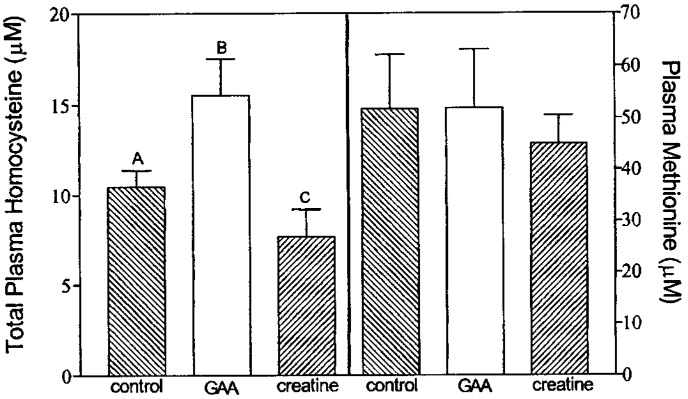

Fig. 2. Effect of GAA and creatine supplementation on total plasma homocysteine (*left*) and methionine (*right*). Rats were maintained on GAA and creatine-supplemented 20% casein AIN 93 diets for 2 wk as described in MATERIALS AND METHODS. Values are means ± SD; $n = 8$ for all groups. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with $P < 0.05$ taken to indicate significant difference. The letters apply to comparisons within each panel. Bars with different letters are significantly different from each other.

enetetrahydrofolate reductase (MTHFR), and betaine-homocysteine *S*-methyltransferase, were assayed in liver. Methionine synthase catalyzes the methylation of homocysteine to form methionine. The methyl donor is 5-methyltetrahydrofolate, which is supplied via the reduction of 5,10-methylenetetrahydrofolate by the enzyme MTHFR. The GAA-supplemented group showed a significant increase (35%) in the activity of methionine synthase compared with either the control or the creatine-supplemented group. Interestingly, the activities of MTHFR among groups show an opposite profile. MTHFR activity is 25% higher in the creatine-supplemented group compared with control animals and 46% higher vs. the GAA-supplemented group. The activity of betaine-homocysteine *S*-methyltransferase, which catalyzes the methylation of homocysteine to form methionine by use of betaine as methyl donor, was unchanged among groups (data not shown).

Table 2. *Effect of GAA and creatine supplementation on selected liver and kidney enzymes*

	Control	GAA	Creatine
Liver enzymes			
Cystathionine β-synthase	5.4 ± 1.3^{ab}	7.2 ± 1.8^{a}	3.8 ± 1.2^{b}
Cystathionine γ-lyase	10.1 ± 2.6	10.3 ± 0.8	10.8 ± 1.7
Methionine synthase	0.23 ± 0.02^{a}	0.31 ± 0.03^{b}	0.23 ± 0.02^{a}
Methylenetetrahydrofolate reductase	0.28 ± 0.07^{a}	0.24 ± 0.03^{a}	0.35 ± 0.04^{b}
Kidney enzyme			
L-Arginine:glycine amidinotransferase	1.1 ± 0.2^{a}	0.3 ± 0.3^{b}	0.2 ± 0.1^{b}

Rats were maintained on control, GAA-, and creatine-supplemented 20% casein AIN-93 diets for 2 wk as described in MATERIALS AND METHODS. Assays were performed on liver tissue except in the case of L-arginine:glycine amidinotransferase, which was assayed on kidney tissue. All activities are expressed as nmol·mg protein^{-1}·min^{-1}. Values are means ± SD; $n = 5$ for all groups. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with $P < 0.05$ taken to indicate significant difference. Data with differing superscripts are significantly different within rows.

Finally, the activity of L-arginine:glycine amidinotransferase, which catalyzes the synthesis of GAA, was assayed in kidney tissue. As expected, GAA and creatine supplementation resulted in a substantial downregulation of enzyme activity. Activity in the GAA- and creatine-supplemented groups was just 27 and 18% of control values, respectively.

Effect of GAA and creatine on homocysteine export from isolated rat hepatocytes. To further characterize the effect of GAA and creatine on homocysteine metabolism, we undertook a series of experiments in which we incubated isolated primary rat hepatocytes in the presence and absence of GAA, creatine, methionine, and serine. The results of these experiments are shown in Table 3. In the presence of methionine, GAA significantly increased the rate of homocysteine export (47% vs. methionine alone), but creatine was without effect. When cells were incubated with methionine plus serine, homocysteine export was decreased by 40% compared with cells incubated with methionine alone. This "serine effect" was also evident in incubations containing methionine plus GAA. When serine was included in these incubations, homocysteine export was lowered by 51%, to the same level of export as the methionine plus serine incubations. These data suggest that alterations in plasma homocysteine imposed by methylation demand may be partly due to effects on liver metabolism.

The objective of this study was to investigate the effects of alterations in methylation demand imposed by GAA and creatine on homocysteine metabolism. GAA would increase methylation demand, and creatine would decrease it. As stated earlier, peripheral L-Dopa methylation by SAM has been shown to elevate plasma homocysteine (1, 23). A similar effect on plasma homocysteine has been observed by Basu and Mann (3) in a study investigating the effects of niacin supplementation on methionine metabolism in rats. Niacin has been shown to exert hypolipidemic effects

Table 3. *Homocysteine export from isolated rat hepatocytes*

Incubation	Homocysteine Export, nmol·mg dry wt^{-1}·h^{-1}
Methionine	2.17 ± 0.17^{a}
Methionine + GAA	3.19 ± 0.22^{b}
Methionine + creatine	2.08 ± 0.23^{a}
Methionine + serine	1.29 ± 0.23^{c}
Methionine + serine + GAA	1.56 ± 0.33^{c}

Cells were isolated as described in MATERIALS AND METHODS and incubated in the presence of the substrates indicated above (all concentrations are 1 mM). Cells were preincubated for 20 min in the presence of medium alone. At the end of the preincubation period, substrates (e.g., methionine) were added, and incubations were allowed to continue for an additional 30 min, after which cells were decanted into Eppendorf tubes and centrifuged for ~90 s. The cell-free supernatant was removed and saved for homocysteine analysis, as described in MATERIALS AND METHODS. Values are means ± SD; $n = 7$ for Met and Met + Ser; $n = 5$ for all others. Data were analyzed by ANOVA followed by the Newman-Keuls posttest, with $P < 0.05$ taken to indicate significant difference. Data with differing superscripts are significantly different from each other.

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when administered in pharmacological doses (10, 15). However, niacin is excreted as methylated pyridones, the formation of which uses SAM as the methyl donor. In their study, Basu and Mann maintained rats on diets supplemented with niacin and monitored effects on plasma sulfur amino acids. Their experiments showed that niacin supplementation significantly increased plasma homocysteine levels. These results, and those of Miller et al. (23) and Allain et al. (1), underscore the importance of peripheral methylation reactions, and the consequent effects on methionine metabolism, during the course of drug therapy.

Creatine biosynthesis begins with the synthesis of GAA in the kidney. L-Arginine:glycine amidinotransferase catalyzes the transfer of the amidino group of arginine to glycine, yielding ornithine and GAA (33). GAA is carried in the blood to the liver, where it is methylated by SAM via guanidinoacetate *N*-methyltransferase to form creatine, which is then exported to extrahepatic tissues. Creatine and creatine phosphate are converted nonenzymatically to creatinine, the excreted form. This loss has been estimated to be ~2 g/day in a 70-kg male (2). Consequently, creatine synthesis must replace that lost as creatinine, and it has been calculated that creatine biosynthesis consumes ~70% of physiologically labile methyl groups, more than all other methylation reactions combined (34).

The principal regulatory site, and rate-limiting step, in the creatine biosynthetic pathway is the L-arginine: glycine amidinotransferase reaction. Creatine exerts feedback repression on L-arginine:glycine amidinotransferase at the pretranslational level, an example of end-product repression (21). This repression permits conservation of the essential amino acids methionine and arginine. Guanidinoacetate *N*-methyltransferase, however, is not subject to this kind of regulation by creatine but rather is dependent only on substrate availability. In light of this, the effect of exogenous creatine would be observed after a period of only days. The effect of creatine under these conditions would be to limit the supply of GAA available for methylation by guanidinoacetate *N*-methyltransferase, thereby decreasing the amount of SAM converted to SAH and subsequently to homocysteine. These expectations were borne out by experiment, in that dietary creatine supplementation downregulated L-arginine:glycine amidinotransferase (Table 2) and decreased plasma homocysteine concentration (Fig. 2). As expected, addition of creatine to hepatocyte incubations had no effect on homocysteine production (Table 3).

We also predicted that provision of GAA would drive creatine synthesis and that the resultant methylation demand would increase plasma homocysteine. Dietary GAA also downregulated the renal transamidinase, presumably via the increased plasma creatine concentration. However, the decreased renal synthesis of GAA is more than offset by the provision of the dietary source of this compound. The increased provision of GAA directly drives increased homocysteine production, as is evident in the experiments with hepatocytes (Table 3). The accumulation of homocysteine in these hepatocyte experiments reflects the fact that the production of this metabolite exceeds its rate of removal by remethylation and transsulfuration reactions. We have previously shown that hepatic homocysteine accumulation is decreased by the provision of serine (28). Serine can affect homocysteine metabolism by acting as cosubstrate for the CβS reaction (Fig. 1, *reaction 5*) and by providing folate-linked one-carbon units, which ultimately provide the methyl donor in the methionine synthase reaction (Fig. 1, *reaction 7*). In light of this, the homocysteine-lowering effect of serine observed in our hepatocyte experiments could be mediated either through enhanced transsulfuration, remethylation, or some combination. Our previous work, however, suggests that, in our hepatocyte model, provision of serine stimulates transsulfuration. At that time we sought to determine the locus of serine action by incubating hepatocytes with L-[1-^{14}C]methionine, which when metabolized through the transsulfuration pathway will give rise to α-[1-^{14}C]ketobutyrate (Fig. 1). α-[1-^{14}C]-ketobutyrate can be metabolized via pyruvate dehydrogenase to yield $^{14}CO_2$. Label in unmetabolized α-[1-^{14}C]ketobutyrate can be released by H_2O_2. Our previous experiments showed that $^{14}CO_2$ was significantly higher in hepatocytes incubated with 1 mM L-[1-^{14}C]methionine and 1 mM serine than in those incubated with L-[1-^{14}C]methionine alone. The increase in $^{14}CO_2$ production was roughly equal to the decrease in homocysteine export seen under the same conditions. These results suggested that the inclusion of serine, in our hepatocyte model, acts at the level of CβS to remove homocysteine. Given that we employed identical conditions in our present work, we believe the homocysteine-lowering effect of serine observed here is due to the provision of the cosubstrate for CβS, the first enzyme in the transsulfuration pathway. The provision of serine also decreases the increased homocysteine production brought about by GAA. This confirms the view that homocysteine accumulation is due to an imbalance between its production and removal. Increased production, brought about by GAA, results in homocysteine accumulation that can be corrected by promoting the removal mechanism.

We are aware of no data on the effect of creatine supplementation on homocysteine metabolism in humans. Creatine is a popular supplement for athletes and bodybuilders and is reported by some to be effective in improving high-intensity exercise performance by enhancing ATP resynthesis as a consequence of increased phosphocreatine availability (8, 14).

It is of interest to compare the creatine ingestion by our rats with the doses typically consumed by humans. Creatine consumption by humans usually involves "loading doses" of 20 g/day for 5 days, followed by a 5 g/day maintenance dose. For a 70-kg person, this amounts to a loading dose of ~0.3 g/kg body wt and a maintenance dose of ~0.1 g/kg body wt. Our rats consumed ~10 g food·day^{-1}·100 g body wt^{-1}. Because the supplemented food contains 0.4% creatine, this amounts to an intake of ~0.4 g/kg body wt. One must take care in making such comparisons between ani-

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mals of such different body weights because of the scaling effect of metabolism (26). We would anticipate that creatine ingestion by humans would interact with homocysteine metabolism in the same manner as it does in rats, but whether or not this would result in a significant decrease in plasma homocysteine has yet to be determined.

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Letter to the Editor

Analytical and biochemical aspects associated with supraphysiological creatine intake

During the past decade, creatine has established itself as an ergogenic aid in sports and has gained increasing attention in medical and lay press as a possible therapeutic agent for various neuromuscular diseases. The available literature mainly covers the short-term effects of creatine supplementation on skeletal muscle function in health and disease [1], while little is known about the efficiency of long-term supplementation and possible side effects. This letter addresses briefly the analytical interference of creatine supplementation on common laboratory tests and examines possible side effects and health risks. Creatine is linked to the arginine, guanidino-compound, one-carbon and homocysteine metabolism, as well as muscular energy metabolism.

1. Creatine ingestion and renal function parameters

Increased intake of creatine can interfere with the determination of creatinine concentrations. First, the increased creatine load is converted to creatinine and increases the daily exretion of creatinine. Secondly, analytical interference of creatine on the different creatinine assays can increase creatinine concentrations due to the aspecificity of the assay. In this regard, the apparent increases in plasma creatinine after creatine administration can be attributed to the in vivo degradation of creatine to creatinine, but also to analytical interference of creatine with the plasma creatinine determinations. Depending on the assay used, creatine can substantially interfere with routine creatinine determination. In the colorimetric Jaffé reaction, creatine can act as pseudochromogen resulting in higher apparent creatinine concentrations. Enzymatic assays, using creatinine-to-creatine conversion in their reaction, are also prone to analytical interference with less predictable deviation from the true creatinine concentration. Using an uncompensated rate-blanked kinetic Jaffé assay [2], we investigated the in vitro interference of creatine on this routine creatinine assay. Serum was spiked with increasing concentrations of creatine (0–10 mg/dl). Fig. 1 illustrates the serum creatinine concentrations in function of the creatine concentration added. Creatine addition significantly increased serum creatinine concentrations between 2.5 and 10 mg/dl ($p<0.05$). Endpoint Jaffé-based methods without rate-blanked kinetic correction are prone to larger deviations from the true serum creatinine concentration.

In animals with renal failure, serum creatinine concentrations were apparently found to increase upon creatine administration without evidence of deterioration of renal failure, whereas in animals with normal renal function similar creatinine concentrations were observed upon creatine feeding [2].

The safety of high-dose creatine supplementation has been questioned. Two case reports of deleterious effects of creatine on kidney function have been published. The first describes an interstitial nephritis in a previously healthy young man after ingestion of creatine [3]. In the second, deterioration of renal function in a young man with underlying focal segmental glomerulosclerosis was observed [4]. Although renal function recovered after cessation of creatine, no causal relationship between creatine ingestion and renal failure can be established on the basis of these case reports. In humans, controlled studies did not reveal adverse effects of creatine supplementation. Poortmans et al. [5–7] investigated intensely renal function in young healthy subjects. No

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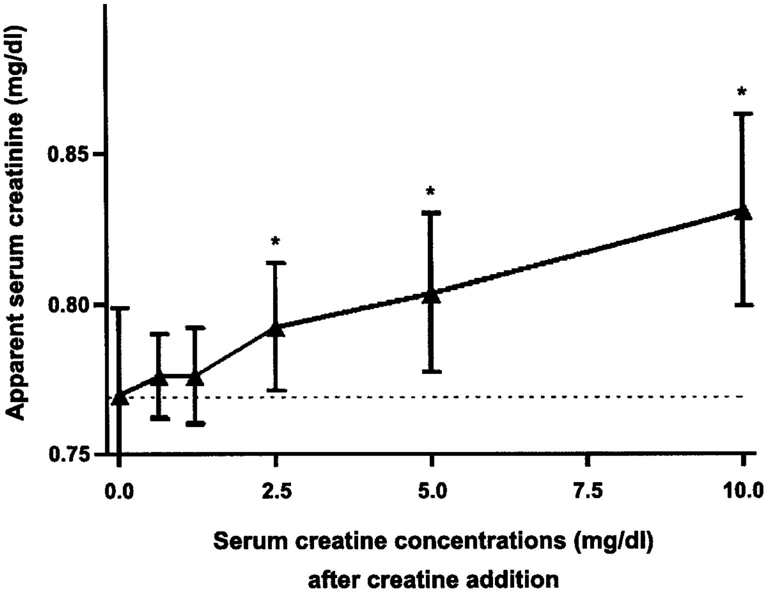

Fig. 1. Apparent serum creatinine concentrations after addition of creatine. *$p<0.05$; serum creatinine concentrations after addition vs. baseline concentrations.

alterations in creatinine, urea or albumin clearance were observed during short-term (5 days) or long-term supplementation (1 month to 5 years) with dosages from 1 to 80 g daily. Robinson [8] and Volek [9] reported only transient increases in apparent serum creatinine during creatine ingestion, without evidence of renal dysfunction. Six weeks after cessation of the creatine supplementation, serum creatinine had returned to baseline [8].

In contrast to humans, experiments in animals suggest progression of renal disease in an animal model of human polycystic kidney disease [10]. In a surgical partial nephrectomy animal model of chronic renal failure, no changes in inulin- or creatinine clearance rates nor in renal protein handling were observed [2]. No biochemical evidence is currently available to assume negative effects of creatine supplementation on liver- or gastroenterological functions.

A possible health risk of creatine, which is insufficiently studied, is the formation of mutagenic compounds. Creatine and creatinine are described as precursors for amino-imidazo azaarenes, a class of food mutagens, found in meat and fish. By heating the meat, creatine can be converted to these mutagenic and carcinogenic compounds. Whether or not high-dose creatine administration is associated with formation of these compounds in vivo is currently unknown [11].

Creatine is classed as a nutritional supplement and is freely available over the counter. These different formulations are not subjected to adequate quality control as with pharmaceutical preparations. Commercially, creatine is synthesized from cyanamide and sarcosine. Toxic side products or contaminants such as dihydrotriazine and dicyanamide could be present in these formulations. As creatine is ingested in high doses, these contaminants could be ingested in sufficient amount to exert toxic side effects. Moreover, both amateur and professional sportsmen tend to use even higher doses then recommended (>20 g).

2. Methylation demand and homocysteine metabolism

A more appealing aspect of creatine supplementation in uremia is the ability to diminish the in vivo methylation demand. Dietary intake (1 g) and endogenous synthesis (1 g) in liver compensate for a daily loss as creatinine of about 2 g. In humans, creatine is synthesized through two successive metabolic steps. Guanidinoacetate is formed in the kidney from glycine and arginine by arginine:glycine aminidinotransferase. Secondly, guanidinoacetate is methylated in the mammalian liver to creatine by guanidinoacetate-methyltransferase with S-adenosylmethionine acting as methyl-donor. Creatine is transported to the muscle in the blood as free molecule and is actively transported into the muscle cell by a specific transporter [11,12]. Methylation of guanidinoacetate accounts for up to 70% of the transmethylation reactions in the body. Quantitatively, this exceeds all other methylation reactions. Stead et al. [12] demonstrated that downregulation of endogenous creatine synthesis by creatine supplementation could decrease methylation demand and homocysteine in animals with normal renal function. In animals with renal failure, creatine supplementation was associated with lower homocysteine concentrations and increased folic acid concentrations, despite similar intakes [13]. However, in humans, a placebo-controlled, cross-over study could not demonstrate any homocysteine lowering potential of creatine supplementation, despite documented uptake of creatine [14]. The effect of creatine on labile methyl pool consumption seems therefore limited in humans.

3. Conclusions

Although creatine is widely used as a performance-enhancing nutritional supplement, safety of creatine administration still remains open for discussion. No major effects on renal function have been described in humans; however, side effects of long-term administration are unknown. Analytical interference of creatine ingestion on creatinine determinations is described and should be taken into account when evaluating renal function. Creatine lowers homocysteine concentrations in animals with normal and diminished renal function, though this effect is not observed in humans. The hype around creatine supplementation is decreasing over the years due to several negative studies on creatine supplementation in various diseases.

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Original Article

Creatine supplementation does not affect kidney function in an animal model with pre-existing renal failure

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Abstract

Background. Creatine is widely used as an ergogenic substance among athletes. Safety of prolonged creatine intake has been questioned, based upon case reports and animal data. We investigated the effect of prolonged creatine ingestion on renal function in animals with normal kidney function or pre-existing kidney failure, respectively.

Methods. Male Wistar rats were randomly allocated to four experimental groups: (i) sham-operated, control diet; (ii) sham-operated, creatine-supplemented diet (2% w/w (0.9 ± 0.2 g creatine/kg body weight/day)); (iii) two-thirds nephrectomized, control diet; and (iv) two-thirds nephrectomized, creatine supplemented diet. Glomerular filtration rate was determined using inulin and creatinine clearance, together with albumin excretion, urea clearance, muscle and serum creatine and serum cystatin C concentrations.

Results. In contrast to previous reports, no detrimental effects of creatine supplementation on the renal function indices were observed in two-thirds nephrectomized or sham-operated animals. No differences were observed in inulin (0.28 ± 0.08 *vs* 0.25 ± 0.08 ml/min/100 g; $P = \text{NS}$) or creatinine clearance rates. Serum cystatin C concentration, urinary protein excretion, and albumin and urea clearance were comparable between creatine-supplemented and control-diet fed animals in both sham-operated and two-thirds nephrectomized animals. Serum creatine and intramuscular total creatine concentrations were higher in creatine-supplemented groups ($P < 0.05$).

Conclusions. Creatine supplementation at a dosage of 2% w/w for 4 weeks does not impair kidney function in animals with pre-existing renal failure or in control animals.

Keywords: creatine supplementation; kidney function

Introduction

In recent years creatine has received considerable attention in the medical and lay press and is widely used as an ergogenic substance. Numerous publications have reported the beneficial effect of creatine supplementation on exercise performance, in healthy subjects or animals as well as in patients or animal models of neurodegenerative diseases [1–3].

As early as 1926, Chanutin [4] reported increased creatinine excretion after creatine ingestion in humans. Several studies have described the non-enzymatic degradation of creatine to creatinine, without evidence of altering kidney function. Phosphocreatine is converted to creatinine at a rate of 2.6%/day and creatine at 1.1%/day *in vivo* [2,4].

Based upon two case reports, safety of prolonged high-dose creatine intake has recently been questioned [5–7], but no causal relationship between creatine ingestion and renal failure can be attributed on the basis of these case reports. Controlled studies [5,8–11] could not demonstrate major adverse effects or health risks of creatine supplementation in humans. Muscle cramps, stomach upset or diarrhoea were reported as sporadic complaints. No biochemical evidence is present on eventual impairment of kidney or liver function in humans. In contrast, in Han:SPRD-cy rats, an animal model of human polycystic kidney disease, creatinine clearance diminished and renal impairment was found to be accelerated upon creatine supplementation [12].

Exact determination of glomerular filtration rate (GFR) in small laboratory animals can be a cumbersome task [13]. Isotope-based methods and inulin clearance are still regarded as gold-standard methods. Creatinine clearance is easier to perform; however, creatinine is partly secreted by the tubules and analytical

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interferences exist in serum creatinine determination. The Jaffé reaction is influenced by protein, bilirubin, glucose and other non-specific chromogens [14]. The magnitude of the interference varies from method to method. Moreover, creatinine production is dependent on body composition and increases upon creatine supplementation. Several low-molecular-weight proteins have been described as good alternatives to serum creatinine in determining kidney function, independent of body composition. Cystatin C is a 13.3 kDa cysteine-protease inhibitor present in plasma. It has been regarded as the product of a 'housekeeping' gene and therefore production is considered to be stable. Cystatin C is freely filtered and neither reabsorbed intact nor secreted. Using anti-human cystatin C antibodies, cystatin C concentrations have been measured in rat sera with good linearity [15]. This study investigates the effects of creatine supplementation on kidney function in rats with normal kidney function and in rats with pre-existing renal impairment. Glomerular filtration was investigated using inulin and creatinine clearances, together with serum cystatin C concentration, 24 h protein excretion, urea and albumin clearance.

Subjects and methods

High-pressure liquid chromatography of the creatine formulation

Creatine monohydrate (99% pure) was obtained from Sigma (St Louis, MO, USA). Possible creatinine contamination of this formulation was assessed by high-pressure liquid chromatography (HPLC) analysis as described previously [16].

Experimental model of moderate chronic renal failure

Male Wistar rats weighing 200–230 g were obtained from Iffa Credo (Brussels, Belgium). The animals had free access to drinking water and were fed *ad libitum*. After acclimatization, animals were randomly allocated to four experimental groups: (i) sham-operated, normal diet ($n=10$); (ii) sham-operated, creatine fed ($n=10$); (iii) renal failure, control diet ($n=12$); and (iv) renal failure, creatine fed ($n=11$). All animal care was in accordance with local prescriptions and the *NIH Guide for the Care and Use of Laboratory Animals*.

Moderate renal failure was induced using a standard procedure of tissue removal (two-thirds nephrectomy), as described previously [17]. In brief, rats were anaesthetized with halothane (Fluothane; AstraZeneca, Södertälje, Sweden) and a flank incision was made exposing the kidney. The upper and lower pole of the left kidney were cryoablated, followed 1 week later by a right nephrectomy. Sham procedure consisted of flank incision and manipulation of the kidney without destruction of tissue. The cumulative mortality, including nephrectomy/sham procedure, was 14%, mostly due to post-operative bleeding or anaesthesia-associated deaths [17]. Renal failure was confirmed by a serum creatinine determination 1 week after the last incision. Dietary manipulation was started 1 week after last surgery/incision. Control animals received a soy-based grounded maintenance chow (RM1; Special Diet Services, Witham, UK) containing 14% protein. Creatine monohydrate (2% w/w) was added to this diet in the creatine-supplemented groups.

Rats were housed in metabolic cages for 24 h during the study at two occasions: at the start and after 4 weeks of creatine supplementation. Urine and blood samples were collected and food intake noted on these occasions. Body weight was measured once a week. Creatine intake in the supplemented groups was calculated from food intake. Creatine intake in the control-diet groups is neglectible due to the absence of meat products in the diet.

Measurement of glomerular filtration rate and proteinuria

Creatinine clearance. Serum and urinary creatinine concentrations were determined using an enzymatic method (Roche Diagnostics, Mannheim, Germany) on a Hitachi 911 Autoanalyzer according to the manufacturer's procedures. Creatinine clearances were calculated based upon 24 h urine collections.

Inulin clearance. After 4 weeks of creatine supplementation, animals ($n=27$) were anaesthetized using pentobarbital (5 mg/100 g) (Nembutal; Sanofi, Libourne, France) and placed on a temperature-controlled heating pad. The trachea was intubated, the left carotid artery was canulated using a PE50 catheter for repeated blood sampling and the left jugular vein was canulated for continuous saline administration at a rate matching diuresis. A single bolus (80 mg/kg) of fluorescein isothiocyanate (FITC)-inulin (Sigma) was administered intravenously and plasma samples were obtained at $t=0$, 3, 30, 120, 140, 160 and 180 min. The FITC-inulin concentration was determined using a 96-well fluorometer (Fluoroscan; Titertek, Huntsville, AL, 35805). Inulin clearance was calculated as described previously [13].

Cystatin C concentrations were measured using anti-human cystatin C antibodies (Dade Behring, Marburg, Germany) on a BN nephelometer (Dade Behring). Relative values are reported, expressed as μg/l of human cystatin C, as described previously [15]. Using this cystatin C assay, correlations between renal function indices were in accordance with published data (serum creatinine *vs* cystatin C: $r=0.92$, $P<0.001$; creatinine clearance *vs* 1/cystatin C: $r=0.94$, $P<0.001$; inulin clearance *vs* 1/cystatin C: $r=0.82$; $P<0.001$). Using these animal samples, the coefficient of variance (CV) value for this assay was 5.0%.

Serum and urine urea were determined enzymatically using Roche diagnostics kits on a modular autoanalyser. Urea clearances were calculated using 24 h urine collections.

Serum albumin was determined using the bromocresol green method. Total urinary protein was measured by pyrogallol red. Urine albumin concentration was determined as described before using a Protur Hisi kit [18].

Tissue collection and processing

After the inulin clearance determinations, the animals were killed by cervical dislocation under pentobarbital anaesthesia

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and hindlimb muscles (soleus and gastrocnemius) were quickly excised, trimmed of fat and connective tissue, washed in phosphate-buffered saline (pH 7.4, 0.075 M), blotted dry, weighed and stored at −70°C for further analysis.

Serum creatine was determined enzymatically [19] and total muscle creatine concentrations were determined colorimetrically [20].

Statistical analysis

Multiple-sample comparison was performed using Kruskal–Wallis testing. One-sample Kolmogorov–Smirnov test was used to pre-test normal distribution. Student's *t*-test (means ± SD) or Mann–Whitney *U*-test [median (interquartile range)] were used to compare separate groups when appropriate. Differences were considered significant at $P<0.05$. Correlation between parameters was examined using regression analysis.

Results

High-pressure liquid chromatography of the creatine formulation

The creatine monohydrate formulation (Sigma) used in this study for supplementation was found to contain 0.37% creatinine. No other side products were detected in the formulation.

Effect of creatine supplementation on glomerular filtration rate

No detrimental effect of creatine supplementation on kidney function could be observed during the study period.

Table 1 summarizes serum and urine renal function indices. Serum creatinine concentrations were higher in two-thirds nephrectomized creatine-supplemented animals, compared with two-thirds nephrectomized control-diet fed animals (0.72 ± 0.19 *vs* 0.58 ± 0.08 mg/dl; $P<0.001$). Sham-operated animals did not differ in serum creatinine concentrations upon creatine supplementation. No significant difference was observed in serum urea or cystatin C concentrations or in 24 h protein excretion between supplemented and control-diet fed animals. Two-thirds nephrectomized animals had significantly different serum creatinine, cystatin C (Figure 1) and serum urea concentrations ($P<0.01$) and exhibited higher 24 h proteinuria [19.3 ± 7.5 *vs* 15.1 ± 4.7 mg; $P<0.05$ (pooled data)] and 24 h albuminuria [5.5 ± 4.5 *vs* 1.9 ± 1.2 mg; $P<0.01$ (pooled data)].

Creatine-supplemented groups exhibited higher serum creatine concentrations [11.66 ± 5.48 *vs* 1.74 ± 1.04 mg/dl; $P<0.0001$ (pooled data)]. No significant differences in creatine concentrations were observed between two-thirds nephrectomized and sham-operated animals for either creatine-supplemented or control-diet groups.

Inulin and creatinine clearance rates, corrected for body weight and serum cystatin C concentrations, are illustrated in Figure 1. No differences were observed in either inulin, creatinine clearance [absolute values (data not shown) or relative to body weight] or serum cystatin C values ($P=\mathrm{NS}$) between creatine-supplemented and control-diet fed animals. Glomerular filtration rate in two-thirds nephrectomized animals was significantly lower compared with sham-operated animals [inulin clearance: 0.21 ± 0.04 *vs* 0.33 ± 0.07 ml/min/100 g, $P<0.001$; creatinine clearance: 0.30 ± 0.07 *vs* 0.48 ± 0.13 ml/min/100 g, $P<0.001$; cystatin C: 97 ± 21 *vs* 50 ± 15 µg/l, $P<0.001$ (pooled data)].

Creatinine clearance rates at the start and after 4 weeks of creatine supplementation are illustrated in Table 2. Creatinine clearance was comparable at the start and after 4 weeks of creatine supplementation between supplemented and control-diet fed rats in either sham-operated or two-thirds nephrectomized animals.

Recuperation of kidney function after renal ablation/nephrectomy was comparable in the creatine-supplemented group *vs* control-diet fed rats (0.69 ± 0.34 *vs* 0.61 ± 0.30 ml/min; $P=\mathrm{NS}$) in the renal failure group.

Table 1. Serum and urine renal function indices after 4 weeks of creatine supplementation

	Sham-operated		Two-thirds nephrectomized	
	Control diet ($n=10$)	Creatine loaded ($n=10$)	Control diet ($n=12$)	Creatine loaded ($n=11$)
Serum creatine (mg/dl)	1.78 ± 0.91	9.21 ± 4.73[a]	1.72 ± 1.20	13.50 ± 5.45[a]
Serum creatinine (mg/dl)[b]	0.43 ± 0.12	0.39 ± 0.15	0.58 ± 0.08	0.72 ± 0.19[a]
Serum urea (g/l)[b]	0.30 ± 0.06	0.32 ± 0.03	0.58 ± 0.12	0.58 ± 0.09
24 h urinary protein excretion (mg/24 h)[b]	16.3 ± 4.4	13.8 ± 4.8	19.3 ± 8.2	19.3 ± 7.3
24 h urinary albumin excretion (mg/24 h)[b]	2.4 ± 1.4	1.4 ± 0.7	5.0 ± 4.2	5.9 ± 4.9

Kruskal–Wallis multiple group comparison was significant for all parameters ($P \leqslant 0.001$).

[a] $P<0.05$, creatine-loaded animals compared with their respective control-diet fed animals.

[b] $P<0.05$, two-thirds nephrectomized animals had significantly different renal function indices compared with their respective controls. Serum creatine did not differ significantly between two-thirds nephrectomized and sham-operated animals.

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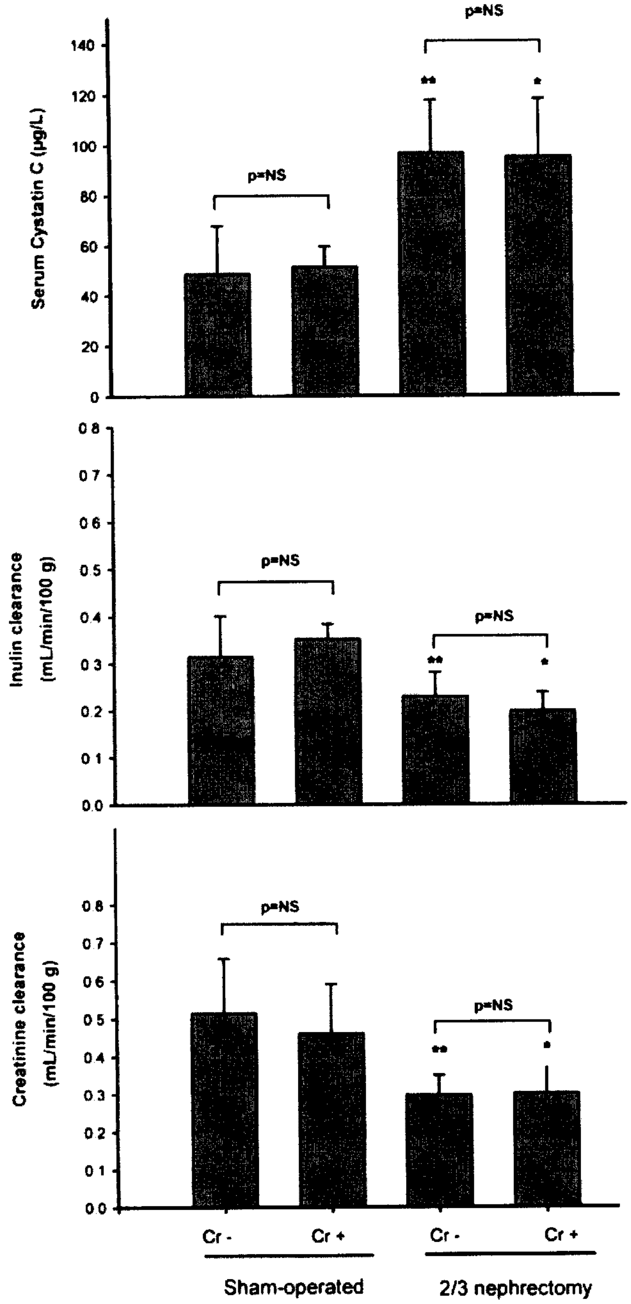

Fig. 1. Effect of 4 weeks of creatine supplementation on inulin, creatinine clearance and serum cystatin C concentrations. No significant differences in inulin, creatinine clearance and cystatin C concentrations were observed between supplemented animals and control-diet fed animals. Two-thirds nephrectomized animals had significantly different glomerular filtration indices compared with sham-operated animals. Cr−, control diet; Cr+, creatine-supplemented diet. $*P<0.05$, two-thirds nephrectomized supplemented animals, compared with supplemented sham-operated animals. $**P<0.05$, two-thirds nephrectomized control-diet fed animals, compared with control-diet fed sham-operated animals.

Effect of creatine supplementation on albumin and urea excretion

Urea and albumin clearance rates are illustrated in Figure 2. No differences were observed in either urea or albumin clearance between creatine-supplemented and control-diet fed animals [urea clearance: 0.21 ± 0.06 *vs* 0.18 ± 0.07 ml/min/100 g, $P=$NS; albumin

Table 2. Creatinine clearance during study period

	Days of creatine supplementation		
	$t=0$ days	$t=28$ days	P-value
Sham-operated			
Control diet ($n=10$)	2.45 ± 0.39	1.96 ± 0.60	NS
Creatine loaded ($n=10$)	2.59 ± 0.26	2.13 ± 0.58	
Two-thirds nephrectomized			
Control diet ($n=12$)	0.54 ± 0.19	1.15 ± 0.22	NS
Creatine loaded ($n=11$)	0.51 ± 0.31	1.17 ± 0.33	

Creatinine clearances are expressed as ml/min. Creatine-supplemented animals did not differ in creatinine clearance rate at the start or after 28 days of creatine supplementation ($P=$NS).

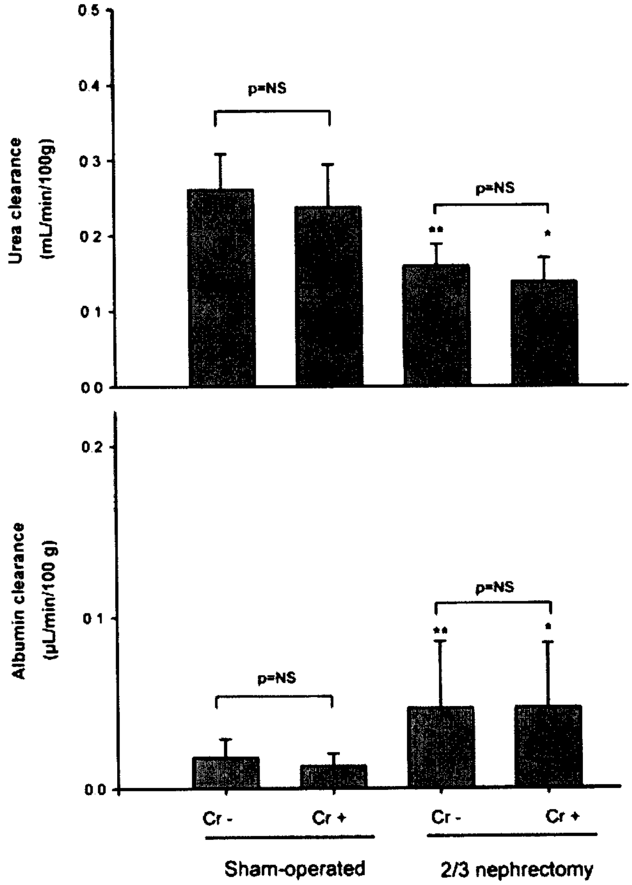

Fig. 2. Effect of 4 weeks of creatine supplementation on urea and albumin clearance rates. No significant differences in urea or albumin were observed between supplemented animals and control-diet fed animals. Two-thirds nephrectomized animals had significantly different urea and albumin clearance rates compared with sham-operated animals. Cr−, control diet; Cr+, creatine-supplemented diet. $*P<0.05$, two-thirds nephrectomized supplemented animals, compared with supplemented sham-operated animals. $**P<0.05$, two-thirds nephrectomized control-diet fed animals, compared with control-diet fed sham-operated animals.

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clearance: 0.019 (0.013–0.036) *vs* 0.018 (0.011–0.042) μl/min/100 g, $P = \text{NS}$ (Mann–Whitney *U*-test) (pooled data)].

Effect of creatine supplementation on body mass, food intake and muscular creatine concentrations

Table 3 summarizes food intake and body mass in the four experimental groups.

No difference in body weight or food intake was observed after 4 weeks of creatine supplementation in either sham-operated or in the two-thirds nephrectomized group.

Two-thirds nephrectomized animals exhibited lower body mass than sham-operated controls [387 ± 22 *vs* 421 ± 21 g; $P < 0.001$ (pooled data)]. Daily food was not different between sham-operated and two-thirds nephrectomized animals [17.5 ± 2.6 *vs* 17.4 ± 3.7 g; $P = \text{NS}$ (pooled data)]. Creatine intake, expressed as absolute intake (353 ± 69 *vs* 333 ± 85 mg; $P = \text{NS}$) or relative to body weight (853 ± 173 *vs* 870 ± 242 mg/kg; $P = \text{NS}$) in supplemented animals did not differ between two-thirds nephrectomized and sham-operated animals.

Total creatine intramuscular concentrations are increased upon supplementation [soleus: 23.9 ± 4.0 *vs* 21.5 ± 2.3 μmol/g, $P = 0.02$; gastrocnemius: 35.8 ± 2.9 *vs* 32.8 ± 1.6 μmol/g, $P < 0.001$ (pooled data)]. No difference in muscular creatine concentration was observed comparing two-thirds nephrectomized animals with sham-operated animals [soleus: 24.0 ± 4.2 *vs* 21.9 ± 2.6 μmol/g, $P = \text{NS}$; gastrocnemius: 34.4 ± 2.6 *vs* 34.5 ± 3.3 μmol/g, $P = \text{NS}$ (pooled data)].

Discussion

The present study could not demonstrate any deleterious effect of creatine supplementation on kidney function. No changes in GFR or renal protein handling were observed. Creatine supplementation was shown to increase intramuscular total creatine concentrations in sham-operated and nephrectomized animals.

Creatinine determinations are susceptible to analytical interferences. These *in vitro* interferences can make up a substantial fraction of the apparent creatinine concentrations. The Jaffé reaction is particularly susceptible to non-specific chromogens, such as bilirubin and cephalosporins [14]. Creatine supplementation can interfere with creatinine determinations by Jaffé's method, with apparent increases in creatinine concentrations up to 8% (Y. Taes, unpublished data). No analytical interferences were found using the enzymatic assay with increased creatine concentrations, as found in animals or subjects who ingest creatine (Y. Taes, unpublished data).

As creatine feeding could interfere with creatinine determination, we investigated GFR by means of the gold-standard procedure (inulin clearance), together with creatinine clearance and serum cystatin C. No significant alterations in any of the markers were observed upon creatine supplementation, indicating no effect on GFR. Urea clearance was comparable in creatine-supplemented and control-diet animals. No changes in 24 h proteinuria or albumin excretion were noted in both sham-operated and two-thirds nephrectomized animals upon creatine supplementation, indicating absence of structural glomerular changes.

Serum creatinine concentrations were found to be higher in nephrectomized supplemented animals but not in creatine-supplemented sham-operated animals. With declining kidney function, increased serum creatinine concentrations are attained in order to excrete the increased creatinine load, due to creatine–creatinine interconversion. However, care is warranted as creatinine has been described as a weak uraemic toxin. Inhibitory effects on metabolic processes have been described, however, at supraphysiological concentrations and not observed *in vivo* [21].

Upon creatine supplementation, Edmunds *et al.* [12] demonstrated renal disease progression in Han:SPRD-cy rats. These authors described increases in serum creatinine concentrations in male (but not in female) rats. Unfortunately, no independent renal function measurements were performed. Creatinine clearances were diminished in both sexes. Creatinine

Table 3. Effect of 4 weeks of creatine supplementation on body mass, food intake and total creatine intramuscular concentration

	Sham-operated		Two-thirds nephrectomized	
	Control diet ($n = 10$)	Creatine loaded ($n = 10$)	Control diet ($n = 12$)	Creatine loaded ($n = 11$)
Body mass (g) ($t = 0$ days)	259 ± 15	262 ± 16	250 ± 11	252 ± 13
Body mass (g) ($t = 28$ days)[a]	416 ± 23	426 ± 18	389 ± 22[b]	386 ± 23[b]
Food intake (g) ($t = 0$ days)	17.3 ± 2.5	18.5 ± 2.0	17.2 ± 3.2	17.8 ± 2.4
Food intake (g) ($t = 28$ days)	17.2 ± 1.3	17.7 ± 3.4	18.2 ± 2.9	16.6 ± 1.2
Total creatine, soleus (μmol/g)	22.1 ± 1.9	25.4 ± 5.0	21.0 ± 2.5	22.7 ± 2.5
Total creatine, white gastrocnemius (μmol/g)[a]	32.2 ± 0.9	35.9 ± 3.5[c]	33.1 ± 1.9	35.8 ± 2.3[c]

Creatine concentrations are expressed as μmol/g wet weight.

[a] $P < 0.05$, Kruskal–Wallis multiple group comparison.

[b] $P < 0.01$, two-thirds nephrectomized animals compared with their respective sham-operated control animals.

[c] $P < 0.01$, creatine loaded animals compared with their respective control-diet fed animals.

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was determined using an end point Jaffé-based method (alkaline picrate; Sigma Diagnostics). The Jaffé method is susceptible to interference from non-specific chromogens [14], varying from method to method. The creatinine values reported by Edmunds *et al.* [12] might have been influenced by creatine concentrations. Unfortunately, no creatine determinations were communicated in the former study. Creatine was supplemented as a mixture of creatine and glutamine in an over-the-counter formulation. This formulation could contain traces of contaminants or toxic products. Purity of this formulation has not been established.

The Han:SPRD-cy rat is a well-established animal model of human polycystic kidney disease. Several interventional studies reported effects on kidney function in this animal. Dietary interventions have been shown to delay the progression of renal disease [22]. While this model mimics human polycystic disease well, it cannot be used as an animal model for general renal functional impairment.

The remnant kidney model is a well-established animal model for kidney function impairment, by means of kidney tissue ablation. Intrinsic renal disease is, however, absent in these animals and the kidney function can partly recover over time. These differences in animal model can account for the differences found between the present study and the study by Edmunds *et al.* [12].

In humans, no evidence from controlled studies is available describing adverse effects of creatine supplementation. Poortmans [5,8,9,11] investigated kidney function in young healthy subjects. No alterations in creatinine, urea or albumin clearance were reported in short-term (5 days, 20 g creatine daily [8]) or long-term studies (1 month to 5 years, with 1–80 g creatine daily [9]; 63 days with 21 g creatine daily [11]). Robinson *et al.* [10] reported only transient increases in serum creatinine during creatine ingestion. Six weeks after cessation of the creatine supplementation, serum creatinine had returned to baseline. No gold-standard procedures for determining GFR, such as inulin or isotope-based methods, have been performed in humans.

Creatine is regarded as a nutritional supplement and available over-the-counter. The different industrially prepared formulations are not subjected to adequate quality control. Toxic side products or contaminants could be present in these formulations. No toxic side products were detected in the creatine monohydrate formulation by our HPLC method [16]. Creatinine contamination was found to be 0.37%. Intake of creatinine is therefore very low in comparison to creatine intake.

This study demonstrates that creatine supplementation can increase intracellular creatine concentration in skeletal muscle of renal failure animals, as described in animals with normal kidney function [1]. Serum and muscle creatine concentrations were found to increase upon creatine supplementation in animals with kidney function impairment. Creatine supplementation in animals has been shown to increase intracellular creatine concentration (total, free and phosphocreatine) in both fast-twitch and slow-twitch skeletal muscles, together with an improved running performance [1].

Differences in creatine concentrations between the soleus and gastrocnemius are in accordance with reported values [1], reflecting differences in energy metabolism. No intramuscular phosphocreatine or high energy nucleotides are reported in the present study as the prolonged anaesthesia during inulin clearance determination could have influenced these high energy metabolites.

In conclusion, we could not demonstrate any harmful effects of prolonged high dose creatine supplementation on glomerular filtration or protein excretion in an animal model with pre-existing moderate renal dysfunction. Muscle intracellular creatine concentration was increased upon creatine supplementation in these animals.

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CHAPTER TWENTY-FOUR

DETECTION OF NITRIC OXIDE AND ITS DERIVATIVES IN HUMAN MIXED SALIVA AND ACIDIFIED SALIVA

Umeo Takahama,* Sachiko Hirota,† *and* Oniki Takayuki*

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Abstract

Nitrate is secreted into the human oral cavity as a salivary component. The nitrate is transformed to nitrite and nitric oxide (NO) by oral bacteria. NO is oxidized by O_2 producing NO_2 and N_2O_3 and also by O_2^- producing $ONOO^-$. Salivary peroxidase can oxidize nitrite and NO to NO_2 or its equivalent in the oral cavity. Nitrite dissolved in saliva is mixed with gastric juice, generating nitrous acid that is transformed to NO and NO_2 via N_2O_3 by self-decomposition. In addition, nitrous acid can react with ascorbic acid and phenolics producing NO

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and with H_2O_2 producing ONOOH. This chapter deals with the detection of reactive nitrogen oxide species (RNOS), especially NO, N_2O_3, NO_2, and $ONOO^-$/ONOOH, in mixed whole saliva and acidified saliva using fluorescent probes and spin-trapping reagents. It is also shown that measurements of nitration and oxygen consumption are useful in studying the formation and scavenging of RNOS in the aforementioned systems.

1. INTRODUCTION

Nitrate is normally added as a food preservative to make ham and bacon, and nitrite is added to the processed food to make the color bright pink due to the formation of methomyoglobin (Benjamin, 2000). Nitrate is also found in large quantities in vegetables such as lettuce and spinach but the quantities of nitrite in vegetable are small. The ingestion of food that contains nitrate and nitrite results in their uptake from intestine and is then secreted as a salivary and urinary component. Nitrate is, in general, nonreactive with organic molecules, but nitrite generated by the reduction of nitrate is reactive. For example, nitrite is a nitrating and nitrosating reagent and an antimicrobial agent especially under acidic conditions (Allaker, 2001; Silvia Mendetz, 1999). In the human oral cavity, nitrate secreted as a salivary component is reduced to nitrite and nitric oxide (NO) by certain bacteria, and salivary nitrite may be transformed to NO, NO_2, and N_2O_3 after mixing with gastric juice. It is well known that NO is an antimicrobial compound as well as a physiologically important compound (Halliwell and Gutteridge, 1999). This chapter deals with the detection of reactive nitrogen oxide species (RNOS) derived from nitrite in the human oral cavity and the species formed in acidified saliva using methods that have been employed in the authors' laboratory.

2. FORMATION OF REACTIVE NITROGEN OXIDE SPECIES (RNOS) IN MIXED WHOLE SALIVA AND THE BACTERIAL FRACTION

The concentration of nitrate in saliva (0.2–2.5 m*M*) is dependent on the amount of nitrate ingested (Pannala *et al.*, 2003; Tanaka *et al.*, 2004). The nitrate in saliva is reduced to nitrite (pK_a of 3.3) and NO successively by bacteria such as *Streptococcus salivarius*, *S. mitis*, and *S. bovis* (Palmerini *et al.*, 2003). The concentration of nitrite in saliva (0.05–1 m*M*) is dependent on the concentration of nitrate (Pannala *et al.*, 2003). The nitrite and NO formed in the human oral cavity can be oxidized by molecular oxygen (reaction 24.1) (Halliwell and Gutteridge, 1999) and by salivary peroxidase (reactions 24.2 and 24.3) (Takahama *et al.*, 2003a, 2006a,b) producing NO_2:

$$2NO + O_2 \rightarrow 2NO_2 \qquad (24.1)$$

$$NO + H_2O_2 \rightarrow NO_2 + H_2O \qquad (24.2)$$

$$2NO_2^- + H_2O_2 + 2H^+ \rightarrow 2NO_2 + 2H_2O \qquad (24.3)$$

Bacteria and leukocytes produce H_2O_2 required for the peroxidase-catalyzed reactions in the oral cavity (Tenovuo, 1989). The NO_2 produced by reactions 24.1–24.3 can contribute to the formation of N_2O_3:

$$NO + NO_2 \leftrightarrow N_2O_3 \qquad (24.4)$$

The production of H_2O_2 shows that $O_2^{-}\cdot$ is also produced in the oral cavity. The production of $O_2^{-}\cdot$ results in the formation of $ONOO^-$ (pK_a of 6.8) if NO is present (Goldstein *et al.*, 2005; Halliwell and Gutteridges, 1999):

$$O_2^{-}\cdot + NO \rightarrow ONOO^- \qquad (24.5)$$

NO_2 is an oxidant as well as a nitrating agent, N_2O_3 is a nitrosating agent, and $ONOO^-$/ONOOH is an oxidant as well as a nitrating and nitrosating agent.

In addition to NO, nitrite, O_2^-, and H_2O_2, SCN^- is also present in human saliva in a concentration range from 0.25 to 2 m*M* (Tsuge *et al.*, 2000). SCN^- is a physiological substrate of salivary peroxidase and inhibits the peroxidase-catalyzed reactions 24.2 and 24.3 producing $OSCN^-$, an antimicrobial agent. Therefore, SCN^- can protect the oral cavity from damages induced by NO_2 and its equivalents if the reactive species are formed by peroxidase-catalyzed reactions. SCN^- is supposed to be produced by the metabolism of isothiocyanates and by the detoxification of cyanide (Galant, 1997; Tsuge *et al.*, 2000).

It happens that the local pH in the oral cavity decreases to about 5 accompanying the growth of acid-producing bacteria (Marsh and Martin, 1999). Under these conditions, NO may be formed from HNO_2 (pK_a of 3.3) (Duncan *et al.*, 1995):

$$2HNO_2 \leftrightarrow N_2O_3 + H_2O \qquad (24.6)$$

SCN^- may interfere with the reaction significantly by the following reaction,

$$SCN^- + HNO_2 \rightarrow NOSCN + OH^- \qquad (24.7)$$

NOSCN is not only an oxidizing but also a nitrosating agent as the compound can dissociate to NO^+ and SCN^- (Benjamin, 2000; Licht *et al.*, 1988). Thus, SCN^- seems to have two functions in the human oral cavity: inhibition of the formation of RNOS and enhancement of their formation.

3. DETECTION OF RNOS IN MIXED WHOLE SALIVA AND THE BACTERIAL FRACTION

To study the metabolism of RNOS in saliva, some kinds of samples may be prepared. They are whole saliva filtrate, bacterial fraction, and salivary peroxidase preparation (Fig. 24.1). In addition, the leukocyte fraction is also useful in studying the metabolism of nitrogen oxides. This section deals with their preparation and then with the detection of RNOS.

3.1. Whole saliva filtrate, bacterial fraction, and salivary peroxidase preparation

Mixed whole saliva can be collected by chewing paraffin or Parafilm as convenient (Takahama *et al.*, 2006a). The saliva (5–10 ml) is passed through two layers of nylon filter nets (32 μm^2) to remove epithelial cells and other particles. The filtrate can be used as a whole saliva filtrate after mixing the filtrate with an equal volume of 50 m*M* sodium phosphate buffer or solutions used for physiological studies. Mixing results in a decrease in the viscosity of saliva and prevention of the increase in pH due to the evaporation of CO_2 gas. The filtrate is centrifuged at 20,000*g* for 5 min. After

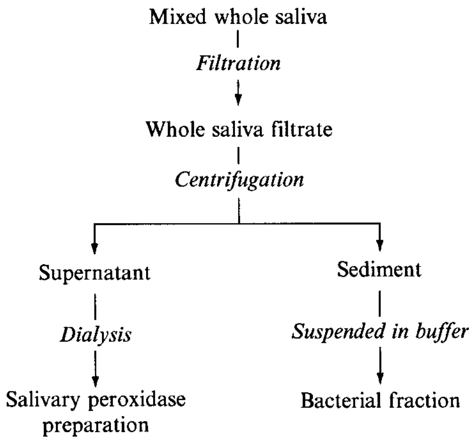

Figure 24.1 Fractionation of mixed whole saliva.

suspending the sediment in a buffer solution, the suspension can be used as a bacterial fraction. The contamination of epithelial cells and leukocytes in this fraction is small. When salivary peroxidase is required for experiments, the supernatant obtained after centrifugation is dialyzed against 10 m*M* sodium phosphate buffer (pH 7.0). The dialyzed supernatant can be used as a salivary peroxidase preparation after removing the turbidity by centrifugation (20,000*g*, 5 min).

3.2. Preparation of leukocytes

The preparation of leukocytes released into the oral cavity has been reported by Yamamoto *et al.* (1991) and by Al-Essa *et al.* (1994) using nylon mesh filtration and centrifugation. Leukocytes are collected by washing the oral cavity with 10 ml of Krebs–Ringer phosphate for 30 s several times. The pooled washings are passed through two layers of nylon filter nets (32 μm^2) to remove epithelial cells. The filtrate is centrifuged at 300*g* for 5 min. After washing the sediment with Krebs–Ringer phosphate or its equivalent, it is suspended in the washing solution. The number of leukocytes is usually adjusted to 1–5 × 10^6/ml.

3.3. Detection of RNOS by nitration of phenolics

The production of RNOS can be measured using some techniques. A classical method is nitration of phenolics. The formation of nitrated compounds suggests the production of NO_2 and/or $ONOO^-/ONOOH$. Tyrosine and 4-hydroxyphenylacetic acid (HPA) (Fig. 24.2) are used to detect the reactive nitrogen species. HPA is found in the human mixed whole saliva (Takahama *et al.*, 2002b) and is nitrated to 4-hydroxy-3-nitrophenylacetic acid (NO_2HPA) in the mixture that contains 0.1 m*M* HPA, 1 m*M* $NaNO_2$, and 0.5 m*M* H_2O_2 in 1 ml of salivary peroxidase fraction (pH 4.5–8) (Hirota *et al.*, 2005; Takahama *et al.*, 2003a). The NO_2HPA formed is extracted with acidic ethyl acetate (20 μl of 1 *M* HCl in 5 ml of ethyl acetate) and is separated by HPLC combined with an ODS column (e.g., Shim-pack LCL-ODS, Shimadzu, Kyoto, Japan). NO_2HPA can be identified comparing the retention time and absorption spectrum

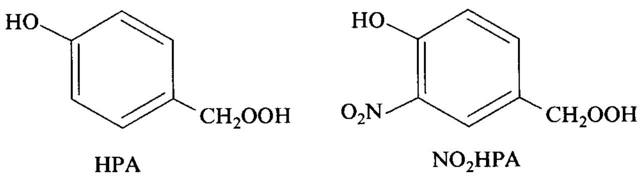

Figure 24.2 Structures of HPA and nitrated HPA.

with standard NO_2HPA. If mass spectra are measured, the identification is more accurate. SCN^- inhibits nitration when salivary peroxidase participates in the nitration. It is interesting to know the formation of NO_2HPA in the human oral cavity. Saliva preparations obtained from about 70 individuals (30–90 years old) with periodontal diseases were extracted with acidic ethyl acetate as described earlier, and NO_2HPA was separated by HPLC. The component was found in the seven saliva preparations whose ages were between 60 and 80. The concentration ranged from 0.03 to 1.7 μM [mean ± SD 0.40 ± 0.65 μM ($n = 7$)] (unpublished result). Data suggest that salivary component HPA could be nitrated in the oral cavity.

Nitration of salivary proteins is also possible in the reaction mixture that contains 1 mM $NaNO_2$ and 0.5 mM H_2O_2 in 1 ml of salivary peroxidase fraction. The nitration of tyrosine residues in proteins is estimated by the increase in absorbance at 420 nm around neutral pH and is confirmed by the detection of nitrated tyrosine by HPLC after hydrolysis of the nitrated proteins (Takahama *et al.*, 2003a). Tyrosine residues in amylase seem to be nitrated (unpublished data).

3.4. Fluorometric detection of RNOS

3.4.1. Background

Some kinds of RNOS can also be detected using reagents that are transformed to fluorescent compounds by reactive nitrogen species. In the reagents, 4,5-diaminofluorescein (DAF-2) is widely used to detect the formation of NO under aerobic conditions (Fig. 24.3). It is supposed that N_2O_3 formed by reaction 24.4 transforms the *o*-diamino group of DAF-2 to a triazol group producing triazol fluorescein (DAF-2T), which is fluorescent (excitation wavelength, 495 nm; emission wavelength, 515 nm) (Nakatsubo *et al.*, 1998). The fluorescence yield of DAF-2T is nearly constant in a pH range from 7 to 12. Other reagents, which can react with N_2O_3, are 3-amino-4-monomethylamino-2′,7′-difluorofluorescein (DAF-FM) (Kojima *et al.*, 1999) and 3-amino-4-monomethylaminorhodamine (DAR-4M) (Kojima *et al.*, 2001). Fluorescence yields of triazol forms of DAF-FM (DAF-FMT) (excitation wavelength, 500 nm; emission wavelength, 515 nm) and DAR-4M (DAR-4MT) (excitation wavelength, 560 nm; emission wavelength, 575 nm) are nearly constant in pH ranges from 5.5 to 12 and from 4 to 12, respectively. Esters of DAF-2, DAF-FM, and DAR-4M are taken up into cells, and the esters can be hydrolyzed by esterase producing DAF-2, DAF-FM, and DAR-4M to react with N_2O_3 in the cells (Kojima *et al.*, 1999, 2001). Although the aforementioned reagents react with N_2O_3 generating their triazol forms, it has been reported (i) that DAF-2 is oxidized by peroxidase and some oxidants to DAF-2 radical, which reacts with NO producing the tirazol form (Espey *et al.*, 2002; Jourd'heuil, 2002), and

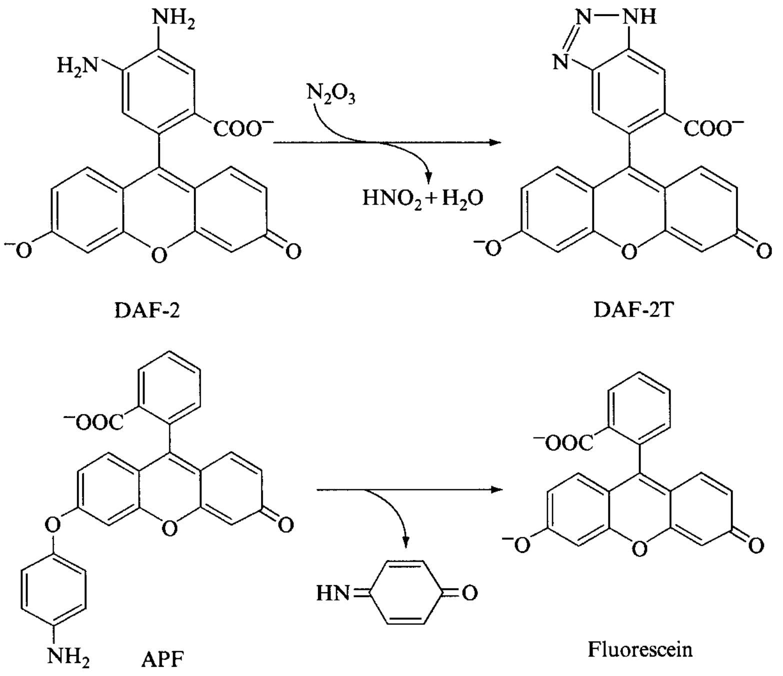

Figure 24.3 Transformation of DAF-2 and APF to their fluorescent components, DAF-2Tand fluorescein.

(ii) that the detection of NO is interfered by ascorbic and dehydroascorbic acids (Zhang *et al.*, 2002).

Other reagents that can be used to detect reactive nitrogen species are aminophenyl fluorescein (APF) (Fig. 24.3) and hydroxyphenyl fluorescein (HPF) (Setukinai *et al.*, 2003). The reagents are ethers of *p*-aminophenol and fluorescein and *p*-hydroxyphenol and fluorescein, respectively. The ether bond of APF is cleaved oxidatively by oxidants such as $ONOO^-$/ONOOH, OH radical, and OCl^-/HOCl, and the bond of HPF can be by $ONOO^-$/ONOOH and OH radical, producing fluorescein (excitation wavelength, 490 nm; emission wavelength, 515 nm). The formation of $ONOO^-$/ONOOH can be detected using dihydrorhodamine 123 (Ischiropoulos *et al.*, 1995). Rhodamine 123 is the fluorescent product (excitation wavelength, 500 nm; emission wavelength, 535 nm) obtained by the oxidation of dihydrorhodamine 123.

3.4.2. Practical

DAF-2T is formed when 10 μM DAF-2 is added to bacterial fraction (pH 7) in the presence of nitrite (Takahama *et al.*, 2005, 2006a,b). N_2O_3 participates in the formation of DAF-2T (Takahama *et al.*, 2006a).

The mechanism of the formation of N_2O_3 by reaction 24.4 is discussed taking (i) the reduction of nitrite to NO by nitrite-reducing bacteria, (ii) autooxidation of NO to NO_2 (reaction 24.1) and (iii) salivary peroxidase-catalyzed oxidation of nitrite and NO to NO_2 (reactions 24.2 and 24.3) into consideration (Takahama *et al.*, 2006a). The participation of salivary peroxidase in the formation of NO_2 is supposed by the result that SCN^- inhibits the formation of DAF-2T in bacterial fraction, which is induced by nitrite and (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR 3), an NO producing reagent (Kita *et al.*, 1994). It is known that the bacterial fraction contains salivary peroxidase and produces H_2O_2 (Takahama *et al.*, 2006a; Tenovuo, 1989). When the concentration of nitrite is increased to about 1 m*M*, the contribution of reactions 24.1 and 24.4 for the formation of N_2O_3 increases due to the increase in the formation of NO by nitrite reducing bacteria. This is supported by the result that the inhibitory effects of SCN^- on the formation of DAF-2T decrease as the concentration of nitrite is increased. The transformation of DAF-2 to DAF-2T by the aforementioned reactions is confirmed by HPLC (Takahama *et al.*, 2005). Not only DAF-2T but also other unidentified fluorescent components can be detected under some conditions. It may be important to investigate the fluorescent species formed, as fluorescent components other than DAF-2T are formed as major fluorescent components. The formation of DAF-2T is also observed in the saliva filtrate, although the rate of formation is slow (Takahama *et al.*, 2005, 2006a). If the formation of DAF-2T is detected by HPLC combined with a spectrophotometric detector, the amount can be calculated using DAF-2 unless standard DAF-2T cannot be obtained because extinction coefficients of DAF-2T ($7.3 \times 10^4 M^{-1}\ cm^{-1}$ at 491 nm) and DAF-2 ($7.1 \times 10^4 M^{-1}\ cm^{-1}$ at 486 nm) are known. Antioxidants in saliva and polyphenols in food inhibit the formation of DAF-2T by scavenging NO_2 or inhibiting the salivary peroxidase-catalyzed oxidation of nitrite (Takahama *et al.*, 2006b).

The local pH in the oral cavity decreases to about 5. Effects of pH on the formation of N_2O_3 could be studied using DAF-FM (Takahama *et al.*, 2007c). The addition of 7 μM DAF-FM to the bacterial fraction in the presence of 0.2 m*M* $NaNO_2$ resulted in a fluorescence increase due to the formation of DAF-FMT. Analysis of the fluorescent product by HPLC showed that the product was the same as that formed by NOR 3 in 50 m*M* sodium phosphate buffer (pH 7.0). As NOR 3 decomposes, producing NO, and the NO is transformed to N_2O_3 by reactions 24.1 and 24.4, the fluorescent product formed by NOR 3 was supposed to be DAF-FMT. The rate of DAF-FMT formation was examined as a function of pH in the presence of nitrite. The result indicated that the rate of DAF-FMT formation increased as pH was decreased. By studying the effects of SCN^- (1 m*M*), it is concluded (i) that SCN^- inhibited the formation of N_2O_3 around pH 7 by inhibiting the salivary peroxidase-catalyzed oxidation of nitrite and NO as described earlier and (ii) that SCN^- enhanced the formation of the component that could contribute to the formation of DAF-FMT around pH 5. NOSCN formed by reaction 24.7 was supposed to be involved in the enhanced formation of DAF-FMT as NOSCN is an NO^+ donating reagent (Benjamin, 2000). The formation of DAF-FMT could be quantified from the decrease in the concentration of DAF-FM, as the decrease in concentration was proportional to the fluorescence increase and DAF-FMT was the major fluorescent product.

The incubation of 10 μM APF with 10 μM NOR 3 in 50 mM sodium phosphate buffer (pH 7.0) resulted in the fluorescence increase due to the formation of fluorescein (Takahama *et al.*, 2007c). The formation of fluorescein could be confirmed by HPLC. As OH radical, $ONOO^-$/ONOOH, and OCl^-/HOCl may not be produced in the reaction system, it was concluded that NO_2 generated by reaction 24.1 could oxidize APF. When the bacterial fraction was incubated with 10 μM APF in the presence of 0.2 mM $NaNO_2$, the fluorescence increase due to the formation of fluorescein was observed. As the fluorescence yield of fluorescein decreased as pH was decreased from 7 to 5 (Lakowicz, 2006), quantification of fluorescein formed was difficult by the measurement of the fluorescence increase. Separation of fluorescein by HPLC made possible the estimation of fluorescein formed at various pH values. The participation of OH radical, NO_2, $ONOO^-$/ONOOH, and OCl^-/HOCl in the fluorescein formation in bacterial fraction can be studied by changing the composition of the reaction mixture and by using scavengers of specific reactive species. The formation of fluorescein could also be detected by the addition of APF to the whole saliva filtrate. Dihydrorhodamine 123 is oxidized to a fluorescent component in the bacterial fraction in the presence of 0.2 mM nitrite (Takahama *et al.*, 2006a). Effects of superoxide dismutase cannot be detected in the reaction mixture, to which inhibitors of salivary peroxidase have not been added. The failure may be because of much faster oxidation of dihydrorhodamine 123 by the peroxidase-dependent reactions than the $ONOO^-$/ONOOH-dependent reaction. The effect of superoxide dismutase can be observed when the salivary peroxidase-dependent oxidation of dihydrorhodamine 123 is inhibited (Takahama *et al.*, 2006a). As salivary peroxidase-dependent reactions, direct oxidation of dihydrorhodamine 123 by salivary peroxidase and oxidation of dihydrorhodamine 123 by NO_2 produced by salivary peroxidase-catalyzed oxidation of nitrite are possible.

3.5. Detection of nitric oxide by electron spin resonance (ESR)

Using the fluorometric methods described earlier, RNOS derived from nitrite and NO are detected. Using the ESR technique, NO itself can be detected, as NO binds to ESR-active NO traps directly. Usage of the traps

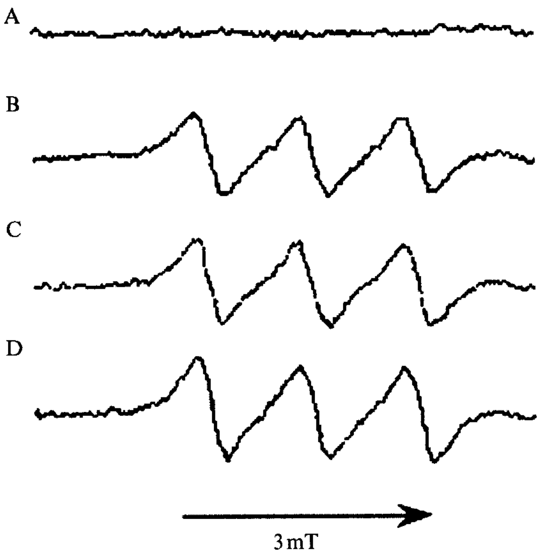

Figure 24.4 ESR spectra of NO-Fe-(DTCS)$_2$. The reaction mixture contained 0.3 ml of 10 m*M* sodium phosphate containing 5 m*M* DTCS, 1.5 m*M* FeCl$_3$, and 0.3 ml of a bacterial fraction. The reaction mixture was incubated for 30 min. A, no addition; B, 1 m*M* NaNO$_2$; C, B + 1 m*M* uric acid; D, B + 1 m*M* NaSCN.

has been reviewed elsewhere (Kalyanaraman, 1996). In the traps developed to detect NO, *N*-(dithocarboxyl)sarcosine (DTCS) (Fujii *et al.*, 1996) has been applied to the bacterial fraction and mixed whole saliva filtrate (Takahama *et al.*, 2005). DTCS has also been applied to detect NO in other biological systems (Plonka *et al.*, 2003; Yoshimura *et al.*, 1996). The formation of NO in the saliva filtrate and bacterial fraction can be detected in the reaction mixture that contains 5 m*M* DTCS and 1.5 m*M* FeCl$_3$. In our study, a mixture of 10 m*M* DTCS and 3 m*M* FeCl$_3$ is prepared in 50 m*M* sodium phosphate buffer (pH 7–7.6), and the mixture (0.5 ml) is mixed with 0.5 ml of bacterial fraction or saliva filtrate. The intensity of the ESR signal of the NO-Fe^{2+}-(DTCS)$_2$ adduct increases as a function of time. The ESR spectrum has three peaks due to a nitrogen nucleus, as shown in Fig. 24.4.

4. FORMATION OF RNOS IN ACIDIFIED SALIVA

Saliva is mixed with gastric juice in the stomach. As the pH of gastric juice is about 2, an event that occurs initially on mixing is the protonation of salivary nitrite ion producing nitrous acid (pK_a of 3.3). Nitrous acid is an active species, and the acid can produce N_2O_3 by reaction 24.6, which transforms to NO and NO_2 by the reverse reaction of reaction 24.4. In the gastric juice, ascorbic acid is contained and the acid can react with nitrous acid:

$$\text{Ascorbic acid} + HNO_2 \rightarrow NO + H_2O + \text{ascorbyl radical} \quad (24.8)$$

In addition to ascorbic acid, some dietary phenolic compounds (e.g., quercetin, caffeic acid, and chlorogenic acid) can also react with nitrous acid producing NO as reaction 24.8 (Peri *et al.*, 2005; Takahama *et al.*, 2002a, 2003c). Once NO is produced, it is transformed to NO_2 and N_2O_3 using O_2 in the mixture of saliva and gastric juice. NO and HNO_2 may react with HO_2 and H_2O_2, respectively, producing ONOOH and ONOOH + H_2O if HO_2 and H_2O_2 are present in the mixture of saliva and gastric juice (Takahama *et al.*, 2007a). Ascorbic acid and phenolics in food are able to scavenge NO_2 and ONOOH. The former scavenges them by reduction and the latter mainly by reduction and nitration.

SCN^- is also a normal component of saliva, and SCN^- can interfere with the ascorbic acid-dependent reduction of nitrous acid and the decomposition of ONOOH. At first, SCN^- can enhance the formation of NO by the reaction between ascorbic acid and nitrous acid in reaction 24.8 (Licht *et al.*, 1988). NOSCN formed by reaction 24.7 may contribute to the enhanced formation of NO:

$$\begin{aligned}\text{Ascorbic acid} + NOSCN \rightarrow NO + SCN^- + H^+ \\ + \text{ascorbyl radical}\end{aligned} \quad (24.9)$$

The enhancement of NO formation by SCN^- is discussed in relation to the rapid decrease in the concentration of O_2 in the mixture of saliva and gastric juice as gastric juice contains ascorbic acid (Takahama *et al.*, 2003c). If NO is transformed to NO_2 and N_2O_3 consuming O_2 in the presence of ascorbic acid, ascorbic acid can reduce the nitrogen oxides to nitrous acid. When the concentration of O_2 decreased, NO formed can diffuse to stomach cells to play its original roles (Brown *et al.*, 1992; Desai *et al.*, 1991; Lundberg *et al.*, 2004; McColl, 2005; Pique *et al.*, 1989).

Nitrous acid can react with H_2O_2 producing ONOOH as described previously if H_2O_2 is produced in or taken up into the stomach. SCN^- in the mixture of saliva and gastric juice can react with ONOOH producing CO_2, NH_3, and SO_4^{2-} and consuming H_2O_2 (Takahama *et al.*, 2007a). In the absence of SCN^-, ONOOH can participate in the nitration and nitrosation of phenolics.

The reaction of nitrous acid with phenolics to produce NO does not seem to be affected by SCN^- (Peri *et al.*, 2005; Takahama *et al.*, 2003c). In the mixture of nitrous acid and chlorogenic acid in the absence of SCN^-, the quinone form of chlorogenic acid and nitrated chlorogenic acid are formed (Takahama *et al.*, 2007b). Nitrous acid itself and NO_2 formed from NO and HNO_2 may oxidize chlorogenic acid to its radicals, which are nitrated by NO_2 or transformed to the quinone by dismutation. By adding SCN^- to the aforementioned reaction mixture, the formation of nitrated chlorogenic acid and the quinone is inhibited and (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*] [1,3]oxathiol-4-yl)acrylooxy)quinic acid is formed via 2-thiocyanatochlorogenic acid (Takahama *et al.*, 2007b). The result suggests that SCN^- can prevent the formation of quinones, which are bioactive compounds (Moridani *et al.*, 2001), by the nitrous acid-dependent oxidation of diphenols in food in the mixture of saliva and gastric juice.

5. DETECTION OF RNOS IN ACIDIFIED SALIVA

5.1. Detection of nitric oxide by oxygen consumption

Nitric oxide produced under aerobic conditions reacts with O_2 producing NO_2 (reaction 24.1). If oxygen consumption by respiration and other reactions is controlled, the NO formation can be measured using an oxygen electrode (Jensen *et al.*, 1997; Venkataraman *et al.*, 2000). In fact, nitrite consumes O_2 in 50 m*M* KCl-HCl buffer (pH 2). The amount of consumption is dependent on the concentration of nitrite, and the rate of consumption is enhanced largely and more largely by ascorbic acid (0.01–0.1 m*M*) and ascorbic acid plus SCN^- (0.1–1 m*M*), respectively (Takahama *et al.*, 2003b). The enhancement by ascorbic acid is because of ascorbic acid-dependent reduction of nitrous acid (reaction 24.8) and NOSCN (reaction 24.9). The oxygen consumption is also observed in acidified saliva that contains nitrite and SCN^-. In this way, the measurement of oxygen consumption is useful in detecting the formation of NO in acidified saliva (Takahama *et al.*, 2003b). The reduction of nitrous acid to NO by phenolics can also be detected with an oxygen electrode (Takahama *et al.*, 2003c).

5.2. Detection of nitric oxide by ESR

The NO trapping reagent $Fe\text{-}(DTCS)_2$ is used to detect NO in acidified saliva (Takahama *et al.*, 2003b). After incubation of acidified saliva (0.5 ml) for defined periods, 0.5 ml of a mixture of 10 m*M* DTCS containing 1.5 m*M* $FeCl_3$ prepared in 0.1 *M* sodium phosphate buffer (pH 7.6) is added. As the $NO\text{–}Fe\text{-}(DTCS)_2$ complex is formed around neutral pH, neutralization of the acidified saliva is required on the addition of $Fe\text{-}(DTCS)_2$. Changes in the concentration of NO in acidified saliva can be detected by measuring the intensity of the ESR signal of $NO\text{–}Fe\text{-}(DTCS)_2$.

5.3. Nitration

A salivary component, HPA, is nitrated when the pH of whole saliva filtrate is decreased to about 2 (Takahama *et al.*, 2002b). The nitrated HPA is extracted with ethyl acetate from the acidified saliva and is quantified using an ODS column as described earlier. The nitration of HPA in acidified saliva suggests that nitration of tyrosine residues of salivary proteins is also possible. The nitration of salivary components may relate to scavenging of NO_2 and other oxidants such as ONOOH and OH radicals in the stomach.

5.4. Detection of NO_2^-/HNO_2, NO_3^-, and SCN^-

It may happen that nitrous acid reacts with H_2O_2 in the stomach producing ONOOH. The production of ONOOH may be estimated by the nitration of phenolics and by the formation of HNO_3. One of the ways to detect the HNO_3 formed is the separation of NO_3^- and NO_2^- with a reversed-phase column using the mixture of 25 m*M* KH_2PO_4 and methanol (4:1, v/v; pH 3) as a mobile phase (Takahama *et al.*, 2007b). The presence of phosphate in the mobile phase seems to be useful in separating NO_3^- from NO_2^-/HNO_2. The ONOOH-induced decrease in the concentration of SCN^- is also detected by the aforementioned HPLC system. During the decrease in the concentration of SCN^-, SO_4^{2-} is produced. The production of SO_4^{2-} can be detected by the increase in turbidity of the reaction mixture in the presence of Ba^{2+}. The aforementioned ions can also be detected using ion-exchange columns.

6. CONCLUDING REMARKS

The formation of NO, NO_2, N_2O_3, and $ONOO^-/ONOOH$ in the oral cavity can be detected using fluorescent probes and ESR trapping reagents, but this chapter did not deal with the detection of O_2^- and H_2O_2, although their production was related to the formation of NO_2, N_2O_3, and $ONOO^-/ONOOH$. A convenient method for the detection of H_2O_2 is the peroxidase-catalyzed oxidation of the substrate and the inhibition by peroxidase inhibitors. Many substrates have been reported to measure the rate of H_2O_2 formation (Halliwell and Gutterridge, 1999; Tenovuo, 1989). In salivary systems, an important characteristic of the substrate used to detect the formation of H_2O_2 is that the peroxidase-catalyzed oxidation is not influenced very much by nitrite, as saliva contains various concentrations of nitrite. For example, oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium by the salivary peroxiadse/H_2O_2 system is affected greatly by nitrite, but the oxidation of quercetin is not (Takahama *et al.*, 2006a). The detection of O_2^- can also be affected by salivary redox components. If rate and mechanism of production of reactive oxygen species and RNOS in the oral cavity are made clear under various conditions, relations between formation of the reactive species and various diseases in the oral cavity may be elucidated. Elucidation of the rate and mechanism of the formation of reactive oxygen species and RNOS in the acidified saliva may also be useful in discussing relations between diseases and the active species in the stomach.

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Stability Data

Name: Creatine Nitrate				Storage:0~30℃					Humidity:<75%				
Date	Standard	Batch NO.	Item		Results								
					0 month	3 months	6 months	9 months	12 months	18 months	24 months	30 months	36 months
Sep.15,2009	25kg/Drum	90910.0	Appearance	Almost white crystalline powder									
Conclusion: Stabile			Inspection	Heavy Metals (ppm)	<10	<10	<10	<10	<10				
				Residue on Ignition (%)	<0.1	<0.1	<0.1	<0.1	<0.1				
				Loss on Drying (%)	0.5	0.5	0.5	0.6	0.6				
			Content Assay (%)		97.5	97.5	97.3	97.3	97.3				
			Operator		Lijuan Chen	Lijuan Chen	Lijuan Chen	Lijuan Chen	Guixin Chen				

High Nitrate Content in Drinking Water: Cytogenetic Effects in Exposed Children

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ABSTRACT. The potential genotoxicity of nitrates and nitrites—contaminants of drinking water that have been implicated in carcinogenesis—was investigated in this study. Sister chromatid exchanges and frequency of chromatid/chromosome aberrations were studied in peripheral blood lymphocytes of 70 children who were 12–15 y of age. These children were permanent residents in geographical areas of Greece, where elevated concentrations of nitrates (i.e., 55.70–87.98 mg/l) existed in drinking water. The control group comprised 20 healthy children who resided in areas with very low nitrate concentrations (i.e., 0.7 mg/l). A significant increase in the mean number of chromatid/chromosome breaks was observed in children exposed to nitrate concentrations that exceeded 70.5 mg/l ($p < .01$), but there was no significant increase in the mean number of sister chromatid exchanges per cell. The results indicate that chronic administration of elevated concentrations of nitrate in drinking water has the capability of inducing cytogenetic effects.

NITRATES (NO_3^-) AND NITRITES (NO_2^-) are natural components of many agricultural products. They are also used widely in food and pharmaceutical industries.[1] These chemical substances can react with amines and amides to produce nitrosamines and nitrosamides in gastric fluid (i.e., *N*-nitroso-compounds [NNCs]).[2] These compounds readily induce tumors of the liver, esophagus, kidneys, intestine, and central nervous and lymphoid systems of laboratory animals.[3] There is evidence that humans are also susceptible to the carcinogenic effect of NNCs, and a relationship between nitroso-compounds and gastric cancer has been postulated.[3,4] The potential mutagenic effects of NNCs have been investigated in only a few in vitro and in vivo cytogenetic studies on laboratory animals.[5–9] The effects of exposure to nitrogen fertilizers in humans, however, have been investigated in only one study, in which a lack of genetic damage was reported.[10]

In the present study, analyses of sister chromatid exchanges (SCEs) and chromosome aberrations were

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performed in lymphocytes of children who resided permanently in agricultural areas of central Greece, where elevated concentrations of NO_3^- were measured in drinking water.

Material and Method

Concentrations of NO_3^-/NO_2^- in drinking water. We sampled drinking water from different agricultural and intensive farming areas of central Greece to determine NO_3^- and NO_2^- concentrations. Water samples were collected at consecutive monthly intervals during a 9-mo period, which commenced in April 1994. No significant differences in NO_3^- values were observed for the samples collected at different monthly intervals. Results reported in this study are the mean of the average of duplicate samples, which were analyzed each month.

Concentrations of NO_3^- and NO_2^- in the water samples were measured, using the cadmium reduction and diazotization methods, respectively.[11] Precision of the analytical determination exceeded 10% for NO_3^- and 20% for NO_2^-, and the respective detection limits were 0.1 mg/l and 0.008 mg/l.

Populations studied. After determination of NO_3^-/NO_2^- concentrations, we focused the cytogenetic investigation on 70 children who were 12–15 y of age. These children had lived 6+ mo in agricultural areas of Larisa district, located in central Greece, and where NO_3^- concentrations ranged from 55.7 mg/l–87.98 mg/l. Twenty additional children who resided in a nonagricultural area of central Greece, where concentrations of NO_3^-/NO_2^- were very low (0.7 mg/l and 0.014 mg/l, respectively), were used as controls. The study officially commenced after governmental and parental permissions to draw blood were obtained. To exclude factors that might influence the interpretation of cytogenetic results, we asked all volunteer students to complete a questionnaire, in which reference was made to smoking habits and dietary consumption of NO_3^--rich foods. All children in our study were nonsmokers who consumed very small quantities of NO_3^--rich foods (e.g., canned food, smoked meat, beer). In addition, the children were not being treated with drugs or vitamin supplements, they had not suffered from recent viral infections, and they had not taken any x-rays during 6 mo prior to the study.

The 70 children who resided in areas with high NO_3^- concentrations were distributed into three groups, by level of NO_3^- in drinking water (Table 1). Group 1 children (n = 33) resided in a town where the mean concentration of NO_3^- was 55.7 mg/l; children in group 2 (n = 18) resided in three different towns in which similar NO_3^- concentrations existed (i.e., 72.44 mg/l, 70.5 mg/l and 72.7 mg/l, respectively); and group 3 children (n = 19) resided in another town where the mean NO_3^- concentration was 87.98 mg/l.

Cytogenetic analysis. The frequency of SCEs and chromosome aberrations (i.e., chromatid and chromosome breaks) was studied in peripheral blood lymphocytes. Lymphocytes, which were grown in T-199 medium (Seromed) supplemented with 5% fetal calf serum (Gibco), were stimulated with phytohemagglutinin (PHA [Seromed]) for 72 h at 37 °C. Two sets of whole-blood cultures were performed. The numerical evaluation of chromosome aberrations and SCEs was performed in a number of metaphases that are accepted readily in most relevant cytogenetic studies.

Table 1.—Distribution of Children, by Group

Population studied	NO_3^- in drinking water (mg/l)	Children		
		n	M	F
Group 1	55.7	33	21	12
Group 2	70.5–72.7	18	8	11
Group 3	87.98	19	12	6
Controls	0.70	20	11	9

Chromosome aberrations in the first set of cultures were studied after Giemsa staining, and they were scored blind. Fifty well-spread metaphases per individual were examined. An aberration was scored as a break if the discontinuity exceeded the chromatid's width.

Differential staining of the sister chromatids was conducted for the second set of cultures by a modification of the fluorescence plus Giemsa (FPG) technique. We used a mixture of 5-bromodeoxyuridine, fluorodeoxyuridine, uridine, and deoxycytidine (10^{-5} M, 4×10^{-7} M, 6×10^{-6} M, and 10^{-4} M, respectively).[12] For each individual, we analyzed, using a Zeiss microscope, 20–30 well-spread and complete metaphases, which were characterized by good-quality SCEs.

Statistical analysis. Statistical evaluation of the results was performed with a two-way analysis of variance. For each of the four variables (i.e., chromatid breaks, chromosome breaks, total number of breaks, and SCEs), we conducted a model analysis of variance, using gender and the group to which each child belonged as independent variables. In addition, we used multiple-comparison tests to determine analytically[13] all existing significant differences between the four groups of children.

Results

In the groups studied, no significant differences in mean values were observed with respect to gender (Table 2). Multiple-comparison tests for chromatid breaks between all groups are shown in Table 3. A significant increase in the mean number of chromatid breaks/cell was observed only in children of groups 2 and 3, compared with controls ($p < .01$). The intergroup mean value analysis revealed a significant difference between children in groups 1 and 2 ($p < .01$) and between children in groups 1 and 3 ($p < .01$). No significant difference was found between mean chromatid values in children of groups 2 and 3 (Table 3).

The results of chromosome break analysis are shown in Table 4. Significant differences were observed in the mean number of chromosome breaks/cell between groups 2 and 3 and the control group ($p < .01$ and $p < .05$, respectively). Intergroup comparison tests revealed significant mean value differences (Table 5) between

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group 1 and group 2 ($p < .01$), group 2 and group 3 ($p < .05$), and between group 1 and group 3 ($p < .01$).

Discussion

Consumption of drinking water that contains elevated concentrations of NO_3^- can eventually lead to endogenous formation of NNCs, many of which are known to be mutagenic. Mutagenicity of NO_3^- was investigated by the sensitive and rapid cytogenetic methods of chromatid/chromosome breaks analysis and SCEs in peripheral lymphocytes of children. The observed increase in the mean number of chromatid/chromosome breaks indicated, at minimum, an association between NO_3^- content in drinking water and chromatid/chromosome aberrations in peripheral blood lymphocytes. Chromosome aberration results similar to ours have been reported in a few in vitro and in vivo studies on laboratory animals. The results of almost all in vitro studies indicate an induction of chromosome aberrations on different cell lines after exposures to NO_3^-. Initially, it was shown that NO_2^- produced chromosome aberrations in human embryonic lung cells in anaphase mitotic recombination on *Saccharomyces* and in base-pair substitution in *Salmonella*. In studies conducted in Japan, increased chromosome aberrations and SCE frequencies were observed in hamster lung fibroblasts treated with NO_2^-.[6,7] In 1987, Luca et al.[8] observed an increase in chromosome aberrations in BSC-1 and HeLa cells grown for 24 h in cultures with NO_2^- doses of 0.265 mg/ml and 0.530 mg/ml, but a dose-effect relationship was not observed.

Table 2.—Analysis of Variance for All Dependent Variables

Source of variation	Dependent variables: Chromatid breaks *F*	*p*	Chromosome breaks *F*	*p*	SCEs *F*	*p*
Group	33.4	< .0005	19.3	< .0005	4.9	< .005
Gender	0.6	NS	0.03	NS	0.01	NS
Group, by gender	0.5	NS	0.71	NS	1.3	NS

Note: NS = not significant

Table 4.—Multiple Comparison Tests for Chromosome Breaks between All Groups ($F = 19.3$, $p \leq .0005$)

Group	Chromosome breaks/metaphase $\bar{x}$	*SD*	Group 1	2	3	Control
1	0.005	0.011	—	$p < .01$	$p < .01$	NS
2	0.053	0.035	—	—	$p < .05$	$p < .01$
3	0.029	0.031	—	—	—	$p < .05$
Control	0.006	0.009	—	—	—	—

Note: NS = not significant

Table 3.—Multiple Comparison Tests for Chromatid Breaks between All Groups ($F = 33.4$, $p = < .0005$)

Group	Chromatid breaks/metaphase $\bar{x}$	*SD*	Group 1	2	3	Control
1	0.031	0.021	—	$p < .01$	$p < .01$	NS
2	0.086	0.024	—	—	NS	$p < .01$
3	0.074	0.039	—	—	—	$p < .01$
Control	0.019	0.12	—	—	—	—

Note: NS = not significant

Table 5.—Multiple Comparison Tests for SCEs between All Groups ($F = .49$, $p < .005$)

Group	SEC/cell $\bar{x}$	*SD*	Group 1	2	3	Control
1	7.461	0.999	—	NS	NS	$p < .01$
2	7.368	0.699	—	—	NS	$p < .05$
3	7.770	0.614	—	—	—	NS
Control	8.408	1.224	—	—	—	—

Note: NS = not significant

In vivo studies. Luca et al.[8] found a significant increase in chromosome aberrations in rat and mouse bone marrows after 2 wk of NO_3^- administration. In an earlier study, an increase in chromosome aberrations was observed in rats and mice treated intragastrically with NO_2^- doses of 1.72–46.66 mg/kg and in rabbits treated with the same doses (but administered daily in drinking water for 3 mo).[9] In humans, Rojas recently showed a lack of detectable genetic damage, evidenced by the absence of significant chromosome aberrations in the lymphocytes of 23 workers exposed to nitrogen fertilizer.[10] The authors attributed the negative results of this report to several factors, including the rapid metabolic decay of some NNCs formed in vivo and to the low NO_2^- level in the blood, both of which resulted from the rapid oxidation of NO_2^- to NO_3^- via oxyhemoglobin.[15]

Very few in vivo studies of SCEs have been reported, and only included laboratory animals treated with a combination of NO_2^- and various chemicals were included in these studies. This fact complicates the interpretation of results on the genotoxicity of *each* of the chemicals administered separately. More specifically, Giri et al.[16] reported a significant increase of SCEs in mice after administration of a combination treatment of metanil yellow (a dye containing a secondary amino group) and NO_2^-.[16] The same results were observed when another secondary and tertiary-amine-containing dye was used in combination with NO_2^- in vivo in mice.[17] The most recent reported originated in 1993

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from Kligerman et al.,[18] who observed a concentration-related increase of SCEs in rats' spleenocytes after they were exposed to drinking water that contained a mixture of pesticides and NO$_3^-$. In our study, we did not observe an increase in SCE frequency in the children exposed to high concentrations of NO$_3^-$ (70.5–87.98 mg/l), compared with the control group. This negative finding could be attributed to the fact that SCEs and chromosome aberrations result from different cellular mechanisms and represent different types of DNA damage.[19]

To our knowledge, the present investigation on the cytogenetic effects of NO$_3^-$ in drinking water is the first performed on a population of children who had no prominent exposure to any other genotoxic agent. If the observed correlation between chromosome aberrations and a high concentration of NO$_3^-$ in drinking water is to be confirmed, additional human studies—along with in vitro NO$_3^-$ exposure of human lymphocytes—should be undertaken.

* * * * * * * * * *

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Mutagenicity, Creatine and Nutrient Contents of Pan Fried Meat from Various Animal Species

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Vikse, R. and P.E. Joner: Mutagenicity, creatine and nutrient contents of pan fried meat from various animal species. Acta vet. scand. 1993, 34, 363-370. – The mutagenic activity in extracts of fried meat from 16 different animal species was studied in *Salmonella typhimurium* TA98. In each experiment, 1 meat sample together with a standard beef sample was fried, and the mutagenicity was expressed relative to the beef sample. All meat samples showed less mutagenic activity than beef. The contents of creatine, creatinine, water, protein, carbohydrate and fat in the meat samples were analyzed, but mutagenicity was not correlated with the concentration of any of these constituents. Beef meat treated with creatinase to remove creatine produced reduced mutagenic activity. Possibly a threshold concentration of creatine is necessary to give a high mutagenic response.

cooking mutagens, Ames test, creatinase.

Introduction

Cooking of proteinaceous food from animal muscle tissue leads to the formation of mutagenic heterocyclic amines. Model studies have shown that creatine and/or creatinine, monosaccharides and free amino acids act as precursors of mutagenic heterocyclic amines, especially the aminoimidazoazaarenes (*Jägerstad et al.* 1991). Panfrying and baking experiments with bovine tissues and studies on meat flavour products show a relationship between concentration of glucose, monosaccharides, creatine and creatinine in the food stuff and the level of mutagenicity in the prepared product (*Laser Reutersward et al.* 1987a, *Laser Reuterswärd et al.* 1987b). Addition of creatine to a meat product before frying resulted in greatly increased yields of mutagenic compounds (*Nes* 1986, *Becher et al.* 1988).

Other important factors influencing the amount of mutagenic compounds formed are the time of and the temperature during frying (*Laser Reuterswärd et al.* 1987b) and the water (*Bjeldanes et al.* 1983) and fat content of the food stuff (*Spingarn et al.* 1981).

Marked progess has been done in understanding the conditions for the formation of mutagenic heterocyclic amines (*Jägerstad et al.* 1991). Still much work needs to be done regarding factors that might influence the yields of mutagenic amines formed in cooked foods. Most of the cooking experiments with meat and offal has been performed with bovine and pork tissues (*Berg et al.* 1990, *Laser Reuterswärd et al.* 1987b). However fried meat other than beef and pork are frequently consumed. The purpose of the work was to study the yields of cooking mutagens produced in meats from different animal species. The mutagenic

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yield was quantified by exposing *Salmonella typhimurium* to extracts from the cooked product in the presence of a metabolizing system. The results have been analyzed for a possible correlation between mutagenic activity and the contents of total protein, carbohydrate, fat, water, creatine and creatinine. Digestion of finely ground muscle with creatinase reduces the creatine content. We have also studied the effect of such partial removal of creatine from beef on the yields of mutagenic activity after cooking.

Material and methods

Chemicals

Aroclor 1254 was purchased from Chem Service, (West Chester, Pa., USA); ATP*; Benzo(a)pyrene B(a)P; Creatinase; DL-Dithiothreitol; Glucose-6-phosphate; Lactatedehydrogenase (EC 1.1.1.27); Pyruvate kinase (EC 2.7.1.40); NADP; NADH; Phosphoenolpyruvate; Sodium orthovanadate; from Sigma Chemical Co. (St. Louis, MO, USA) and Creatininase (EC 3.5.2.10) from Boehringer Mannheim GmbH (Germany). All other chemicals and bacteriological media were of best quality from standard sources.

The pan frying procedure

Fresh or freshly frozen meat was bought at the local supermarket. The meat free from visible fat and connective tissue, was minced and fried without any additives. Fresh beef from a common shoulder sample from a single animal was used in all the comparative frying experiments as a standard. In the experiments studying the influence of creatinase digestion on the production of cooking mutagens during pan frying beef meat from the leg (3 different animals) was used. The following experimental conditions were maintained to ensure equal treatment of all meat samples during frying: In each experiment two 50 g portions of minced beef and two 50 g portions of minced meat from another animal were formed into patties, 7 cm in diameter. They were placed on a metal plate in 4 separate metal cylinders, to keep the meat separated and to aid transfer into the pan simultaneously. A teflon coated frying pan on a standard hot plate was used for frying. The frying pan was lightly greased with maize oil. The temperature in the frying pan was measured with a termocouple (Digitron, –50+750°C), and when the temperature reached 250°C all 4 pieces of meat patties were transferred into the pan at the same time. To ensure optimal contact between the meat and the hot surface of the frying pan, a lid weighing 400 g was applied on top of the patties. To compensate for possible uneven heating by the plate the pan was rotated 4 times during the 12 min frying period (6 min on each side). The frying was performed with the same hot plate setting each time. The meat was lightly charred.

Extraction and fractionation

The meat crust was freed from the core material with a soft knife and the mutagenic basic organic components were extracted according to the method of *Felton et al.* (1981): The crust was homogenized in acetone (Ultrathorax, Inter Med, Disp 25, Roskilde, Denmark) and filtered through a sintered glass funnel. The extract was cooled to –18°C overnight and then filtered through a Whatman No. 1 filter and concentrated on a rotary evaporator to near dryness.
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Abbreviations: ATP, Adenosine 5'-Triphosphate; DMSO, dimethyl sulphoxide; MeIQx, (2-amino-3, 8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline; NADH, nicotinamide adenine dinucleotide reduced form; NADP, nicotinamide adenine dinucleotide phosphate.

dryness. The filtrate was diluted in 0,01 N HCl and extracted 3 times with CH_2Cl_2. The pH of the aqueous phase was adjusted to pH 12 and again extracted 3 times with CH_2Cl_2 to obtain the basic components This basic extract was concentrated to near dryness on a rotary evaporator and dried under a stream of nitrogen gas. For mutagenicty testing the residues were dissolved in dimethyl sulphoxide (DMSO).

Mutation Assay

Mutagenicity was determined with *Salmonella Typhimurium* TA98 and enzymatic activation (S9) as described by *Maron & Ames* (1983), and measured in triplicate with 20 ml agar per dish.

Dose-response curves in these experiments are assumed to be linear, and the values are therefore based on single dose measurements. The S9 liver-extract was prepared from Aroclor treated rats and the amount of protein per dish was 2 mg. The bacterial strain *Salmonella thypimurium* TA 98, was provided by Dr. Bruce Ames, University of California. Benzo(a)pyrene was used as positive control. The mean number of spontaneous revertants was 39 (SD ± 9) during the experimental period and this value was subtracted from the results shown.

Creatine/Creatinine Analysis

Creatine and creatinine were extracted from meat samples, and measured according to methods described by *Wahlefeldt & Siedel* (1985) with some modifications.

The essay was performed in a 0.2 M glycine/KOH buffer pH 8.0 containing the following compounds: Glycine, 154 mM; Dithiothreitol, 1.5 mM; $MgCl_2$, 23 mM; ATP, 1.0 mM; NADH, 0.35 mM; Phosphoenolpyruvate, 1.0 mM; Pyruvate kinase, 19 kU/l; Lactate-dehydrogenase, 10 kU/l and Creatininase, 7 kU/l. A crude preparation of Creatine kinase (EC 2.7.3.2.), prepared from rabbit muscle (*Keutel et al.* 1972; *Kuby et al.* 1954), was added in adequate amounts. This enzyme preparation was found more suitable than the commercial one. Sodium orthovanadate was added to a final concentration of 0.38 mM, in order to minimize ATP-ase activity in the creatine kinase preparation. The measurement was performed in a Hewlett Packard 8452A diode array spectrophotometer at 340 nM and standards were always included.

Creatinase treatment of meat

50 g minced meat was homogenized for 2 min in 45 ml 0.1 M KCl (Ultrathorax). The pH was adjusted to 7.5 with 1 M Tris base. 1000 U Creatinase was added and the mixture was incubated for 2 or 2.5 h at 37°C in a water bath. The total volume of added liquid was 50 ml. Another 50 g portion of meat was similarly treated but in the absence of enzyme. After incubation, 10% potato starch was added to give the product a suitable texture for frying. Four portions of this dough, 2 with and 2 without enzyme, each weighing 50 g were fried simultanously as described. The meat crust in 2 experiments and both the crust and core in 1 experiment were extracted and fractionated as described.

Food analysis

The content of fat, glucose, nitrogen and water in the meat were performed according to the methods of Nordic committee on food analysis. (*Anon.* 1974 a, b; 1976; 1978).

Results

In the present study, we measured the mutagenic activity extracted from the frying crust of minced meat samples from 16 different animal species in the Ames *Salmonella* test. The

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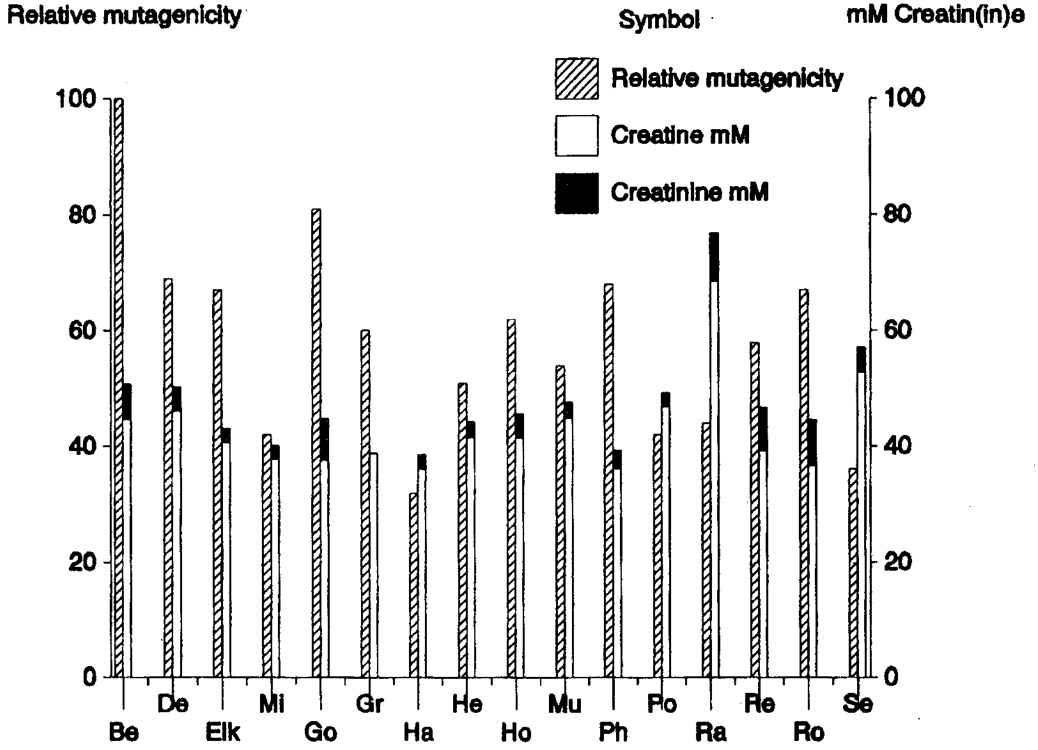

Figure 1. Relative mutagenicity in frying crusts of meat samples from 16 different animal species, compared to the concentration of creatine and creatinine in samples from the same animals. Mutagenicity was determined by the Ames/Salmonella test and related to the mutagenicity of the beef sample fried in the same experiment, which was set to 100 percent. Creatine and creatinine content were determined in uncooked meat. Abbreviations: Be = beef; De = deer; Mi = minced elk; Go = Goat; Gr = grouse; Ha = hare; He = hen; Ho = horse*; Mu = mutton; Ph = pheasant; Po = pork; Ra = rabbit; Re = reindeer*; Ro = roe-deer*; Se = seal*. The results represent the mean value from two identical meat portions fried in the same experiment, except for the species labelled *, where 2 independent experiments were performed. Creatine and creatinine values represent mean of 2 independent experiments. B(a)P as positive control (5 µg/dish) gave 482 ± 168 revertants/dish troughout the study.

results are expressed as percent of the activity extracted from the standard beef samples fried in the same experiment (Fig. 1). In all experiments the common reference sample of beef contained the highest mutagenic activity. Of the other meat products seal gave the lowest score (36% of the standard beef) and goat the highest (81% of standard). The absolute values as *Salmonella typhimurium* TA98 revertants/100 g raw meat are presented in Table 1, accompanied by the values obtained with the standard beef sample that day. The average number of *S. typhimurium* revertants was 30608 ± 6594 (n = 15), expressed per 100 g raw beef.

In Table 2 the content of water, fat, protein and carbohydrate in the 16 different meat samples are presented. The minced elk sample is a commercial meat product that contains 8.8% fat. The correlation coefficients between mutagenicity, and the content of water, fat, protein or carbohydrate were calculated by linear regression to R = –0.265, –0.045, –0.352, –0.313, respectively.

Table 1
vertrants/

Animal
Deer
Elk
Elk, minc
Goat
Grouse
Hare
Hen
Horse
Mutton
Pheasant
Pork
Rabbit
Reindeer
Roe-deer
Seal

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Table 1. Mutagenicity in frying crusts (TA98 revertrants/100 g raw meat) †.

Animal	Meat	Beef
Deer	12140	17540
Elk	23640	35300
Elk, mince	9560	22740
Goat	30420	37460
Grouse	19840	32940
Hare	11260	35200
Hen	20200	39860
Horse	17180	27600
Mutton	15940	29460
Pheasant	22480	32960
Pork	15040	36200
Rabbit	12080	27280
Reindeer	21260	37090
Roe-deer	15610	23200
Seal	8630	24290

† The table presents the mutagenicity of the different meat extracts together with the standard beef sample fried in the same experiment.

Table 2. Content of water, fat, protein and carbohydrate in 16 meat samples from different species (percent) **.

Animal	Water	Fat	Protein	Carbohydrate
Beef	74,1	5,1	20,5	0,1
Deer	70,5	2,0	24,8	0,2
Elk	74,4	1,8	23,1	n.d.
Elk, mince	69,8	8,8	21,4	n.d.
Goat	79,2	1,6	20,0	0,7
Grouse	70,5	2,0	24,6	n.d.
Hare	76,2	1,5	21,8	0,2
Hen	73,4	3,0	23,9	n.d.
Horse	74,3	4,0	21,3	0,6
Mutton	74,2	3,3	22,9	n.d.
Pheasant	74,0	1,4	24,1	n.d.
Pork	75,1	1,3	22,3	n.d.
Rabbit	73,2	1,8	24,2	0,2
Reindeer	71,6	1,9	27,3	n.d.
Roe-deer	74,0	1,8	23,0	0,2
Seal	70,5	2,0	27,3	n.d.

** The analysis were performed according to the methods of Nordic committee on food analysis. n.d. = not detected.

The concentration of creatine and creatinine in the raw meat samples are included in Fig. 1. Creatinine concentration is much lower than the creatine concentration in all meat samples, pork has the lowest concentration with 2,5 mM and rabbit the highest with 8,3 mM. Creatine concentration is lowest in hare with 36 mM and highest in rabbit with 69 mM. The correlation coefficient between mutagenicity and meat content of creatine and creatinine was calculated to $R = -0.138$.

In Fig. 2 the effect of creatinase treatment of beef meat on the extractable mutagenic activity in fried patties is presented, in relation to the creatine and creatinine contents in the meat before frying. The average decrease in creatine concentration was 65% which resulted in 73% average decrease in the mutagenic response from the meat ectract.

Discussion

The total mass amount of mutagens formed during cooking of meat is influenced by cooking temperature and time *(Berg et al.* 1990, *Dolora et al.* 1979, *Felton & Knize* 1991, *Laser Reuterswärd et al.* 1987b). Our mutagenicity value of 30 608 revertants/100 g initial raw weight for beef is higher than results from other studies, where 6300-23500 revertants per 100 g raw weight were obtained for beef patties fried at 200°C for 3-12 minutes *(Bjeldanes et al.* 1982, *Commoner et al.* 1978, *Felton et al.* 1981, *Laser Reuterswärd et al.* 1987b). Interlaboratory variation could also be attributed to variability in the Ames test, in the activity of the S9 preparation, in the source of meat or cooking procedure used *(Felton et al.* 1981).

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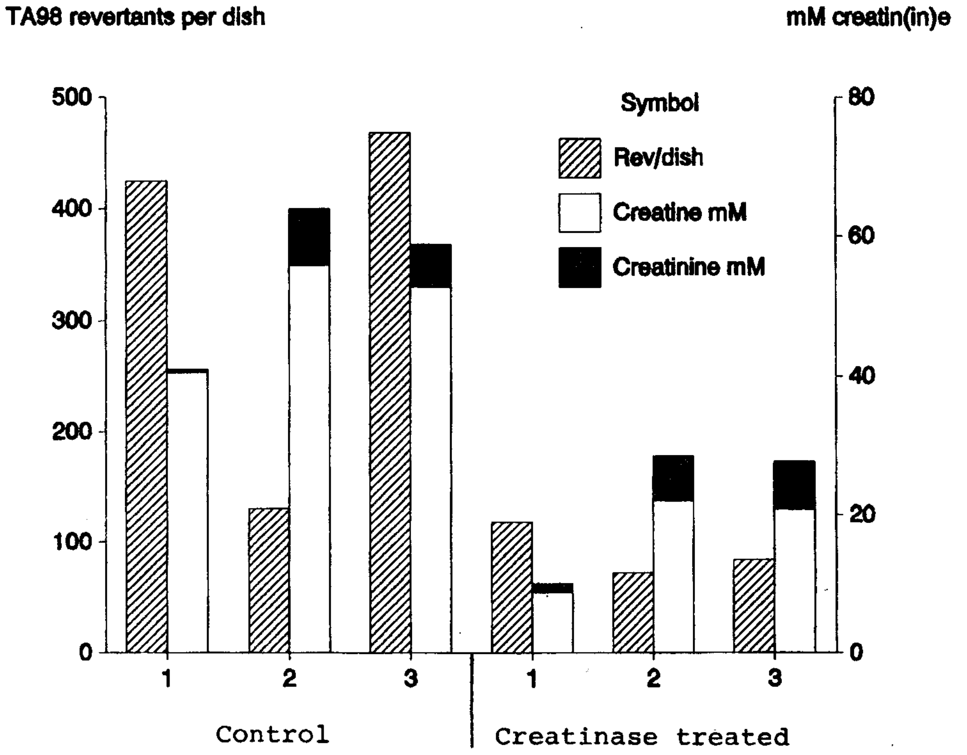

Figure 2. Mutagenicity in extract from fried minced beef treated with creatinase, expressed as Salmonella revertants per dish and creatine and creatinine concentration determined in the uncooked samples. Results from three different experiments with controls are shown. In experiment 1 and 2 mutagens from the crust were extracted, in experiment 3 mutagens from the core and crust were extracted. MeIQx (2-amino-3, 8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline as positive control gave 67 341 ± 8730 revertants/µg (n = 3).

We preferred to cook our meat at a somewhat higher temperature than normally used in the household, to ensure an efficient induction of mutagenic activity in the fried meat patties. Otherwise the cooking conditions and equipment were similar to those normally used in the household. *Felton et al.* (1988) have shown that the same set of mutagenic compounds is produced in meat cooked at 200°C and at 300°C. Care was taken to fry all meat samples under identical conditions, but because of the variability between experiments we decided to express the mutagenicity of the different meat samples relative to a beef sample that was included in all experiments.

The water, fat, protein and carbohydrate content of the different types of meat used in these experiments did not correlate with mutagenic activity produced during cooking.

Several reports have shown that addition of creatine to meat before frying greatly increases the mutagenic activity produced *(Nes* 1986, *Becher et al.* 1988). However, differences in the normal creatine and creatinine content of the meat studied here, do not seem to explain the varying mutagenic response from extracts of the fried meat.

It is known from the literature that when fish is prepared and cooked in the same way as beef, a much lower mutagenic activity is produced *(Felton & Knize* 1991). We measured the creatine and creatinine concentrations in cod to 39 and 0,6 mM, respectively, and these values are very similar to the content of these substances in beef.

Fig. 1 demonstrates that creatine and creatinine concentrations differ considerably between the meat samples from the various species analyzed in this experiment, and they are both lower and higher than the concentrations found in the standard beef sample.

The creatinase experiments of Fig. 2, confirm the suggested relationship between creatine concentration and mutagenicity. However, even if creatine/creatinine are important parameters in mutagen formation, the relationship is not a simple one. This is shown by the lack of correlation between creatine/creatinine content and mutagenicity in Fig. 1. The result of the creatinase experiment also suggests that if a workable procedure could be found for removal of creatine from animal tissues, reduced content of cooking mutagens might be achieved. However, the levels of creatine and creatinine in our meat preparations are after creatinase treatment almost at the same level as that found in bovine heart and tongue samples, as reported by *Laser Reutersward et al.* (1987b). These authors found heart and tongue to contain higher mutagenic activity than muscle when fried under identical conditions. They argued that a possible explanation may be that the relative concentrations of creatine and free amino acids versus dipeptides and monosaccharides in heart and tongue, are different from those in muscle, where the concentrations of creatine, free amino acids and dipeptides are almost equimolar *(Laser Reutersward et al.* 1987b).

Both in cooked foods and in model systems creatinine is still present after heating, and only a small part is used in the reaction *(Laser Reutersward et al.* 1987b, *Skog & Jägerstad* 1990). According to *Jägerstad et al.* (1991) creatinine is an essential precursor in mutagen formation, but its concentration seems not to be a yield limiting factor.

Our results suggest that in meat a certain threshold concentration of creatine is necessary to produce a high mutagenic response. Within the normal concentration range found in the different meats studied, however, there seems to be no correlation between creatine concentration and mutagenicity.

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Sammendrag

Innhold av stekemutagener kreatin, kreatinin og næringsstoffer i ulike typer av stekt kjøtt.

I stekeskorpen på kjøtt dannes det mutagene aromatiske heterosykliske aminer. Vi har stekt kjøttkaker fra 16 ulike kjøttslag og målt den mutagene aktiviteten i ekstrakt fra stekeskorpen med Ames' test. I hvert eksperiment ble to kjøttkaker stekt sammen med to standard kjøttkaker av okse, og mutagenisiteten er uttrykt relativt i forhold til okse. Alle kjøttprøvene gav noe lavere mutagen aktivitet enn okse. Det var ingen korrelasjon mellom mutagenisitet og kjøttprøvenes innhold av kreatin, kreatinin, vann, protein, karbohydrat og fett. Oksekjøtt behandlet med kreatinase gav redusert mutagen effekt. Det er mulig at et visst minimumsnivå av kreatin er nødvendig for å få høy mutagen aktivitet i stekeskorpe fra kjøtt.

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Heat-induced mutagen formation from creatine and fat-soluble constituents of food

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It is now well established that dietary mutagens are formed in a variety of foods as a result of thermochemical processes associated with cooking. Examples of this include charred surfaces and smoke condensates of fish and meat (Nagao et al., 1977), commercial beef extract (Vithayathil et al., 1978) and fried hamburger (Commoner et al., 1978). In addition to thermal conditions, the chemical composition of the food itself affects the formation of heat-induced mutagen formation. One of the several factors which influence the formation of mutagens in fried meat is fat content. When cooking hamburger with lean meat it was necessary to add a certain amount of fat for maximum mutagen formation, and based on this finding, it was suggested that fat might play an important role in mutagen formation in fried beef (Spingarn et al., 1981). The results of our investigation reported in this paper clearly show that creatine, creatinine and other amino acids are capable of reacting with several fat-soluble compounds – particularly vitamins – to produce significant levels of mutagenic activity.

Heat induced reactions between the amino compounds and various fat constituents were carried out using a model system which has been used to study browning reactions (Koehler et al., 1969). In this procedure the reactants are heated in ethylene glycol, a high boiling neutral solvent which allowed the reactions to take place under homogeneous conditions while minimizing the loss of mutagens due to volatilization. 500 μmoles each of the reactants were added to ehylene glycol:water (9:1) in a 125-ml Erlenmeyer flask and heated for 90 min on a hot plate kept at 200°C. From this reaction mixture the basic mutagenic compounds were extracted as outlined under the footnote to Table 1 and assayed for mutagenic activity against *S. typhimurium* strain TA98 in the presence of Aroclor-induced rat-liver microsome (S9) preparation according to the standard Ames procedure (Ames et al., 1975).

The mutagenic activities of the reaction products of creatine with various fat-soluble food components are presented on Table 1. The mutagenicity values reported here are the slopes of the linear portion of the dose–response data which

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represent the number of revertant colonies produced by 1 μmole of the original reactant(s). The ethylene glycol:water mixture when heated alone does not produce mutagens. In control experiments where creatine or the other test compounds were heated alone the level of mutagenic activity detected was either very small or none at all. However, creatine reacts with several of these fat-soluble compounds to yield high levels of mutagenic activity. Among these vitamin K_1 is the most active, yielding values considerably higher than that obtained in a similar reaction between creatine and glucose. Lesser, but still significant, levels of mutagens are formed when creatine is heated with vitamin D, carnitine, vitamin A, betaine, cholesterol and choline. No significant increases in mutagenic activity were noticed in the reaction products of creatine with lecithin or vitamin E.

Since vitamin K_1 reacted with creatine to yield exceptionally high mutagenic activity, we carried out further reactions with this compound against creatinine,

TABLE 1

MUTAGENICITY OF HEATED SOLUTIONS OF CREATINE WITH FAT-SOLUBLE FOOD CONSTITUENTS

Test compound	Mutagenicity (TA98 with S9) Number of revertant colonies/μmole of reactant(s)	
	Compound heated alone	Heated with creatine
Glycol:water control	0	–
Creatine control	4	–
Vitamin K_1	0	173
Vitamin D	0	28
Carnitine	2	26
Vitamin A	0	21
Betaine	1	20
Cholesterol	0	10
Choline	0	8
Lecithin	0	3
Vitamin E	0	2
Glucose	0	83

All test compounds used in this study were obtained from Sigma Chemical Company, St. Louis, MI. Erlenmeyer flasks containing 500 μmoles each of the respective compound or their mixtures in 20 ml ethylene glycol:water (9:1) were heated at 200°C for 90 min. After cooling, the reaction mixture was diluted with 200 ml 1 N HCl. It was extracted once with 100 ml methylene chloride and the organic layer discarded. The aqueous layer was adjusted to pH 11 with sodium hydroxide and the basic mutagens were extracted twice with 100-ml aliquots of methylene chloride. After removal of traces of moisture by filtering through anhydrous sodium sulfate, the methylene chloride extracts were taken to dryness, under vacuum at 40°C. The residue was taken up in DMSO and aliquots representing 2, 10 and 50 μmoles of the original reactant(s) were assayed for mutagenic activity.

arginine, threonine and glycine. The results shown on Table 2 clearly show that while glycine yielded only moderate activity the other 3 amino compounds reacted with vitamin K_1 to produce mutagens at significant levels.

Two principal mechanisms suggested to explain the formation of heat-induced mutagen formation during cooking are the pyrolysis of amino acids or proteins at high temperatures (Nagao et al., 1977a; Matsumoto et al., 1977; Yamaizumi et al., 1980) and browning reactions between sugars and nitrogenous compounds at moderate temperatures (Spingarn et al., 1979; Yoshida et al., 1980a, b; Powrie et al., 1981; Stich et al., 1981; Shibamoto et al., 1981; Wei et al., 1981; Aeschbacher et al., 1981; Matsushima et al., 1981). There may be other possible mechanisms involved in the formation of this class of dietary mutagens which may not require the presence of sugars or pyrolytic conditions. As mentioned earlier, there is evidence that mutagen formation in fried beef is affected by fat content (Spingarn et al., 1981). Our own results (unpublished) clearly show the presence of unidentified thermally unstable constituent(s) in beef suet which can react with creatine, creatinine and a few other amino acids to produce mutagens. Based on these considerations, as well as on the basis of the results presented in this paper, it is reasonable to suggest that reactions of creatine, creatinine or other amino acids with fat-soluble compounds – particularly vitamins – may also play a role in the formation of some of the dietary mutagens. At present we are carrying out chemical identification studies to establish the relationship between the mutagens formed in the model system reported here and those formed in cooked meat.

Acknowledgements

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TABLE 2

MUTAGENICITY OF HEATED SOLUTIONS OF VITAMIN K_1 WITH AMINO COMPOUNDS

Amino compound	Mutagenicity (TA98 with S9) Number of revertant colonies/μmole reactant(s)	
	Amino compound heated alone	Heated with vitamin K_1
Creatinine	3	87
Arginine	1	70
Threonine	8	38
Glycine	1	5

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